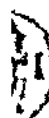
Vol. 46, 1971

Acta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology



S. Karger · Basel · München · Paris · London · New York · Sydney

S. Karger Basel München Paris London New York Sydney
Arnold Böcklin Strasse 25 CH-4000 Basel 11 (Switzerland)

Printed in Switzerland by National Zeitung AG, Basel

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The Value of the Platelet Adhesiveness Test in the Assessment of Abnormalities of Platelet Function

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Abstract. A slightly modified Salzman technique for platelet adhesiveness is described and a normal range of 32% to 65% has been established. There is no variation with age or sex. But investigation shows that this type of technique is sensitive to variations in the packed red-cell volume of the blood, and that for comparable estimates the PCV must be between 33% and 47%. The platelet adhesiveness test gives a low result in von Willebrand's disease and low results have also been found in 4 syndromes characterized by defective platelet aggregation. The reasons for correlation of platelet adhesiveness and platelet aggregation, the limitations of the use of the platelet adhesiveness as a screening test, and the possible role of a plasma factor are discussed.

Key Words
ADP in platelets
Glanzmann's disease
Platelet adhesiveness
Platelet function
von Willebrand's disease

Introduction

By the term 'platelet adhesiveness' (PAd) is usually meant the difference in platelet count before and after native or anticoagulated blood has been in contact with a glass surface. The difference is usually expressed as a percentage of the platelet count before the contact.

Wright [31] was the first who introduced a method for the measurement of the PAd; she used anticoagulated blood placed in a glass flask which was rotated. Moolten and Vroman [16] used a column of glass wool as contact surface instead of the flask. In 1960 Hellem [9] replaced the column of glass wool by one of glass beads (glass bead filter) and used a syringe pump by means of which anticoagulated blood was pushed through the filter. O'Brien's technique [20] differs from that of Hellem's mainly because blood without anticoagulant is used. Later

SALZMAN [27] replaced the syringe pump by a vacutainer, he also used native blood and a glass bead filter

The principle of the method is that the platelets when they come in contact with glass surface adhere to it However, the PAd test does not only count the platelets that adhere to glass HELLEM [8] and SALZMAN [27] have observed that after the passage of the blood through their filter, numerous platelet aggregates are found stuck on the glass surface, indicating that the PAd also counts the platelets which participate in the formation of these aggregates The hypothetical explanation of the formation of these aggregates is that during the blood flow through the filter platelets adhere to the glass and sooner or later other platelets stick to the fixed ones and on each other and finally the platelet aggregates are formed A similar sequence of events is known to happen *in vivo* when a blood vessel is transected platelets from the circulating blood adhere to the damaged surface of the vessel within 2–3 sec and soon other platelets adhere to those already there, finally a platelet aggregate is formed, which is large enough to stop the bleeding from a small vessel

Although there are similarities between the *in vitro* PAd test and the early phase of *in vivo* haemostasis (adherence of platelets to a 'foreign' surface, and formation of platelet aggregates) there are essential differences as well, the main one being that *in vitro* the glass is the 'foreign' surface where the platelets initially adhere instead of the collagen fibrils of the damaged vessel wall Therefore it is not certain to what extent factors which influence the *in vitro* test represent those operating *in vivo* However, the PAd has been used by many workers in the last decade for the investigation of bleeding disorders and hypercoagulable states It appears that it is reduced in von Willebrand s disease [15, 22, 27, 28, 33] and in other platelet functional abnormalities [3, 7, 9, 11, 21, 24, 27, 29] and increased in hypercoagulable states [12, 14, 18, 23, 32] Excellent reviews of the clinical significance of the PAd have recently been published by HARTMANN [8] and HELLEM and STORMORKEN [10]

In the present study problems associated with the technique of the PAd test are described and slight modifications of SALZMAN s original technique are proposed The results of this test in a group of normal persons and of patients with various kinds of platelet functional abnormality are presented The relationship between the PAd and other platelet function tests and the factors influencing the PAd are discussed

Methods

Platelet Adhesiveness Test

SALZMAN's original technique was slightly modified. The glass bead filter was prepared as follows. A vinyl tubing 25.5 cm in length, ID 3 mm (Becton-Dickinson – BD – cat. No 6235) was sealed at one end by a double-Luer plastic adaptor (obtained from discarded Capon Heaton giving sets, cat. No 102, each set having 2 such adaptors one at each end of the rubber tubing). A nylon mesh (Henry Symon Ltd., mesh 25T1) was cemented with Bostick to the end of the adaptor facing the beads. The tubing was filled with glass beads (Jencons No 8, washed according to HELLEM [9]) by means of a glass funnel, and then a rotamixer (Hook & Tucker Ltd.) was used for 10 sec to pack down the beads. The other end of the tubing was stoppered by a Vacutainer Luer adaptor (BD cat. No 5731) also sealed by nylon mesh. The total weight of the glass beads in each filter is found to be 2.7 ± 0.1 g. To the outer end of the Vacutainer Luer adaptor a Vacutainer Holder (BD cat. No 4873) was screwed. A 7 ml Vacutainer was used (BD cat. No 4739) containing 9 mg of dry EDTA (The Vacutainers were supplied in tins of 50; from each tin, when opened, 2 or 3 Vacutainers were tested to check the volume sucked in, which ought to be 7 ± 0.5 ml. If the vacuum was faulty the whole tin was discarded).

The procedure of the test was as follows. A 20 G needle (BD) was connected to the outer end of the double-Luer adaptor of the filter. Cuffed venepuncture was used and a vein with freely flowing blood was selected. Immediately after the venepuncture the Vacutainer was pressed to release its vacuum and a stop-watch started. At 40 sec the tourniquet was released and at 45 sec the filter was disconnected from the needle and a plastic syringe was connected instead, in order to obtain a sample of blood to serve as control. A platelet count was determined from the blood of the control sample and another from the Vacutainer, and the difference between them, expressed as percentage of the control count was the PAd.

The glass bead filters are easily prepared, kept at room temperature and used usually within 2 months after their preparation. Each filter is used only once.

The following are the major methodological factors affecting the results.

Standard filter and procedure. The needle must be 20G, a narrower or wider one gives unreliably high or low results respectively. A standard filter is of utmost importance: a tubing longer or narrower gives higher values and vice versa. The nature and size of the beads is also important (when beads smaller in size were used high PAd values were obtained). An adequate vacutainer vacuum is also essential: when less than 6.0 ml, very high values were obtained; when we tried to re-evacuate the vacutainers the results were unreliable.

The vein. A clean venepuncture from a large vein with a free flow of blood is essential.

The haematocrit (PCV). In figure 1 the mean of the PAd of 151 persons (in whom no platelet dysfunction was present) grouped according to PCV levels is shown. One may observe that the trend of the means of the PAd follows a sigmoid curve. In particular the mean remains constant for the range of PCV between 35 to 45% (statistically the difference of the means is highly [illegible]

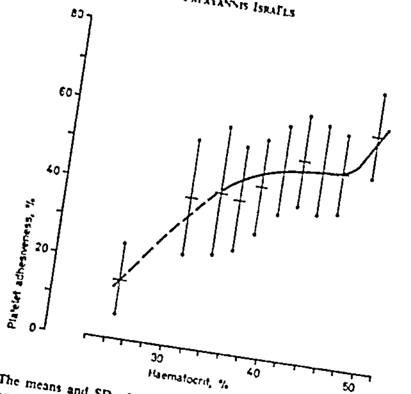


Fig 1 The means and SD of the PAd at various levels of haematocrit (PCV ... The observations are grouped in intervals of 2% is in the range of PCV between 33–46% and the remainder in larger intervals

cant), whilst it rises or falls when the PCV is more than 46 or less than 39%, respectively. For the range of PCV between 33 to 38%, where the mean PAd is reasonably close to the non variable ones we made the following corrections

For PCV	Increase of the PAd value of
33–34%	7%
35–36%	4%
37–38%	2%

It is known that variations in the blood PCV and the blood viscosity are correlated. Consequently a high blood PCV results in a slow blood flow through the filter and therefore a relatively small quantity of blood is collected in the vacutainer at the end of the test. The volume of blood in the vacutainer is quite constant (4.5–5.0 ml) for low PCV levels (38%), but thereafter it becomes progressively smaller as the PCV rises. However, if for any reason (high PCV, narrow vein, unsuccessful venepuncture or vacutainer of low vacuum) the volume of blood in the vacutainer is less than 3.0 ml the PAd value is unreliably high and we ignore the result of the test.

The estimated variation of the PAd was ±7.6%. when the platelet counts were performed in duplicate it was ±5.0%.

For the platelet counts, phase contrast microscopy was used.

The methods of estimating platelet aggregation, ADP release from platelets, platelet factor 3 availability (PF3a) and clot retraction (CR) are described elsewhere [24]. For the bleeding time (BT) Ivy's method was used.

Cases

Normal persons. Staff of this department, students, patients of our clinic who had no bleeding history and their normal relatives were tested over a period of 18 months: total 133.

Platelet function abnormalities. Forty-seven patients who had various kinds of platelet dysfunction were tested. They were classified as follows:

1. Von Willebrand's disease: 27 patients. Diagnostic criteria of this disease were: (a) a life-long haemorrhagic diathesis, (b) autosomal dominant pattern of inheritance (where family history existed), (c) a prolonged bleeding time found at least on one occasion, (d) a low level of the plasma factor VIII, (e) reduced PAd, and (f) platelets normal in number, morphology and function as tested by the methods of aggregation, ADP release, platelet factor 3 availability and clot retraction.

2. A mild haemorrhagic diathesis characterised *in vitro* by prolonged BT, reduced PAd, and also by delayed adherence of platelets to collagen fibrils, absence of aggregation with collagen, abnormal aggregation with thrombin and reduced ADP release from platelets. In the blood films abnormally large platelets are seen. Five patients with this diathesis have been seen, 4 of them belonging to one family with surname Windle, so we refer to this diathesis as 'Windle defect'.

3. A life-long haemorrhagic diathesis characterised *in vitro* by prolonged BT, absent or reduced ADP release from the platelets, absence of the second wave of aggregation with ADP (but normal aggregation with 1.0 M f. c. of ADP), normal or partial aggregation with collagen, normal adherence of platelets to collagen fibrils and occasionally impaired PF3a. Ten patients with this diathesis have been seen, 5 of them belonging to one family with surname 'Hughes', so we refer to this diathesis as 'Hughes defect'.

4. Glanzmann's disease: two patients described elsewhere [25].

5. Sprowson anomaly: in this condition there is a life-long bleeding tendency, the platelets show abnormal aggregation with ADP (they do not change shape and do not disaggregate following the addition of small amounts of ADP) and there is *impaired ADP release*. We have seen only one patient so far, and full details are given elsewhere [24].

Results

Normal Persons

One hundred and thirty-three normal persons were tested, 86 females and 47 males. Figure 2 illustrates the distribution of the PAd results. The mean value for both sexes was 47.95% (SD 9.47), for males

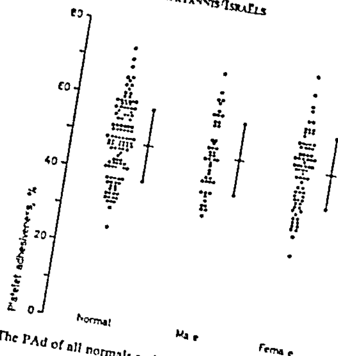


Fig. 2. The PAd of all normals and of the male and female separately.

47.91% (SD 9.81) and for females 48.02% (SD 9.47). The difference of the PAd in males and females is not significant ($P<0.01$). By applying statistical analysis of variance no variation of PAd with age was found. In 6 normal persons in whom the test was performed on 2 occasions at different times, the PAd showed no significant variation.

Platelet Functional Abnormality

Figure 3 shows that the PAd of various kinds of platelet dysfunction is reduced compared with the normal.

In von Willebrand's disease the PAd was reduced. The mean value was 24.1% (SD 12.04), compared with the normal the difference is significant ($P < 0.01$). In some cases in which the test was repeated the results varied widely and not infrequently were found within the normal range, however the PAd was never more than 50%.

All the cases with Windle defect had at least on one occasion a low PAd, but an occasional result within the lower normal limits was found in 3 of them.

In Hughes defect the PAd was abnormally low in 5 patients, within the lower normal limits in 3 and high in 2.

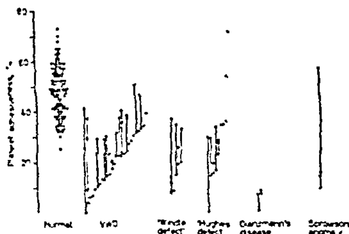


Fig 3 The PAd in various platelet functional abnormalities compared with normal. Each vertical line represents 1 patient and each dot 1 test.

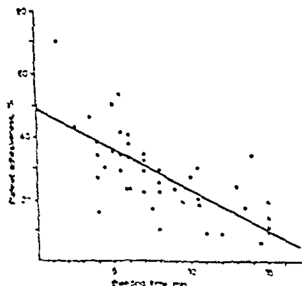


Fig 4 Relationship of PAd and BT in the various platelet functional abnormalities. The line represents the regression line of PAd on BT (y = 47.8 1.28x).

In Glanzmann's disease both patients had twice a very low PAd

In Sprowson anomaly the patient had 3 times low PAd and in one occasion normal

Relation Between PAd and BT in Platelet Functional Abnormality

In figure 4 the diagram shows that there is an inverse correlation between the PAd and the BT of the various platelet dysfunction cases (number of pairs, 45, r =−0 77) In cases in which the tests were repeated the finding of a higher PAd was usually associated with a shorter BT In normal persons there was no correlation between BT and PAd

Relationship Between PAd and Other Platelet Function Tests

Normals Two groups of normal persons, one (10 cases) with PAd > 55% and the other (10 cases) with PAd < 40% were tested for aggregation in final ADP concentrations of 1 0 and 20 0 μM/ml, with collagen, adrenalin 5 0 μM/ml f c and thrombin 1 0 U/ml f c for the release of ADP from platelets after aggregation, for PF-3a and CR The result were similar in both groups

Platelet functional abnormality In von Willebrand s disease the other platelet function (PF) tests were normal Variation of PAd in the same patient was not associated with any change in PF tests

In one case of Windle defect on one occasion when the PAd was found normal a very weak aggregation with collagen was present and the ADP-release was within the lower normal limits, the PAd was found very low in the same patient 3 times when the aggregation with collagen was nil and there was no ADP release at all In the other cases variation of the PAd was not associated with changes in PF tests

In the Hughes defect it seems that the PAd is related to the amount of ADP which is released from platelets One patient clinically unaffected, with high PAd (70%) and ADP-release within the lower normal limits (1 0 μM) Three cases who had no ADP release at all had a low PAd in 2 of them when the tests were repeated although the ADP release was again zero, the PAd was found higher – but still below the normal – and the BT shorter In all the rest the ADP release was very low (0.5 μM) and the PAd varied from low to normal values (usually within the lower normal limits)

In Hughes defect some other abnormalities are occasionally found in the aggregation test, partial aggregation with collagen and disaggregation

following the aggregation with ADP in a final concentration of 20.0 µM/ml, (which never occurs normally with that amount of ADP). The PAd showed no significant change in the same patients when, on different occasions, these tests were found normal or abnormal.

In both cases of Glanzmann's disease the PAd was twice found very low and the aggregations were always abnormal.

In the case of Sprowson anomaly, when the PAd was found low, the response to and release of ADP were abnormal. On one occasion in which the PAd was high the platelets were found to release a normal amount of ADP (2.0 M), in the aggregation with 1.0 µM ml of ADP they showed a normal change in shape and a very weak disaggregation was present.

Discussion

The Method

The Salzman technique has several advantages: the use of native instead of anti-coagulated blood means that the test is carried out in conditions closer to normal. It also provides a brief contact time of blood with the glass so that early changes in the blood are revealed, and the rapid flow of blood through the filter enables a good differentiation between normal and pathological states. The technique is easy and simple.

The modification we use differs from the original Salzman method mainly in that a greater amount of glass beads (2.7 g instead of 1.0 g) and a longer filter are used. These differences have the following sequences: (1) A greater glass surface. (2) A longer contact time of the blood with the glass which results in a higher PAd figure [4], our normal range was 32–65%, compared with 26–60% of SALZMAN's. (3) A slightly slower rate of flow of the blood through the filter. This makes our modification compared to that of SALZMAN more sensitive in detecting differences of increased PAd [1] but less sensitive in differentiating the normal from von Willebrand's disease [22] and probably from other conditions with decreased PAd. However, in our method a quite rapid blood flow is provided resulting in good differentiation between the normal and all the kinds of platelet function abnormality including von Willebrand's disease.

In our modification the PAd is not influenced by the PCV when the latter is between 39 to 46% (which includes the greater proportion of the normal population) but it is gradually reduced when the PCV de-

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In one case of Windle defect on one occasion when the PAd was found normal a very weak aggregation with collagen was present and the ADP-release was within the lower normal limits; the PAd was found very low in the same patient 3 times, when the aggregation with collagen was nil and there was no ADP-release at all. In the other cases variation of the PAd was not associated with changes in PF tests

In the Hughes defect it seems that the PAd is related to the amount of ADP which is released from platelets One patient, clinically unaffected, with high PAd (70%) and ADP-release within the lower normal limits (1.0 µM) Three cases, who had no ADP-release at all had a low PAd, in 2 of them, when the tests were repeated, although the ADP-release was again zero, the PAd was found higher – but still below the normal – and the BT shorter. In all the rest the ADP-release was very low (0.5 µM) and the PAd varied from low to normal values (usually within the lower normal limits)

In Hughes defect some other abnormalities are occasionally found in the aggregation test, partial aggregation with collagen and disaggregation

following the aggregation with ADP in a final concentration of 20.0 μM/ml, (which never occurs normally with that amount of ADP). The PAd showed no significant change in the same patients when on different occasions, these tests were found normal or abnormal.

In both cases of Glanzmann's disease the PAd was twice found very low and the aggregations were always abnormal.

In the case of Sprowson anomaly, when the PAd was found low, the response to and release of ADP were abnormal. On one occasion in which the PAd was high the platelets were found to release a normal amount of ADP (2.0 M), in the aggregation with 1.0 μM/ml of ADP they showed a normal change in shape and a very weak disaggregation was present.

Discussion

The Method

The Salzman technique has several advantages: the use of native instead of anti-coagulated blood means that the test is carried out in conditions closer to normal. It also provides a brief contact time of blood with the glass, so that early changes in the blood are revealed, and the rapid flow of blood through the filter enables a good differentiation between normal and pathological states. The technique is easy and simple.

The modification we use differs from the original Salzman method mainly in that a greater amount of glass beads (2.7 g instead of 1.0 g) and a longer filter are used. These differences have the following sequences: (1) A greater glass surface. (2) A longer contact time of the blood with the glass, which results in a higher PAd figure [4], our normal range was 32–65%, compared with 26–60% of SALZMAN's. (3) A slightly slower rate of flow of the blood through the filter. This makes our modification compared to that of SALZMAN more sensitive in detecting differences of increased PAd [1], but less sensitive in differentiating the normal from von Willebrand's disease [22] and probably from other conditions with decreased PAd. However, in our method a quite rapid blood flow is provided resulting in good differentiation between the normal and all the kinds of platelet function abnormality, including von Willebrand's disease.

In our modification the PAd is not influenced by the PCV when the latter is between 39 to 47%, (which includes the greater proportion of the normal population) but it is gradually reduced when the PCV de-

creases from 38 to 33% and it is unreliably low or high for a PCV less than 33% or more than 46% respectively. A correction of the PAd according to the PCV was made in the range of the latter between 33 to 38%. When we used a slightly different technique, in which the blood was passed in a faster rate through our filter by means of a driven syringe, the PAd was related to the PCV throughout the range of the latter in a linear manner.

With Salzman's original method we found very low PAd values in normal persons who had a PCV just below 40%; we found that this limitation could be overcome by lengthening the filter.

Application of the Results in Platelet function-Abnormalities

The results obtained in patients with various defects of platelet aggregation show that the PAd is also reduced in all of these defects, so PAd reflects the whole process of the formation of the platelet aggregate. Reduced PAd has been reported in von Willebrand's disease by many authors who used the Salzman's technique. Others, using techniques which provide a slow flow of blood through the filter did not find any difference between normal and von Willebrand's disease [5, 9, 20]. Low values for PAd have also been reported in Glanzmann's disease [10, 27] and in other cases of abnormal platelet function [3, 7, 11, 21, 29].

Another test which is abnormal in all the platelet functional abnormalities is the BT. We found that there is a correlation between the BT and the PAd, which is easily explained by the fact that in the course of the BT, platelet aggregates are formed and seal the bleeding capillaries, thus, any defective platelet aggregate formation results in a prolonged BT. Many other reports suggest that there is a correlation between PAd and BT [2, 6, 9, 13, 17, 27].

The finding of a prolonged BT or reduced PAd indicates that a platelet defect is present, but does not tell us the nature of the defect; for determining the particular defect the platelet aggregation tests must be carried out. Used together the PAd and the BT provide a useful indicator because each gives a qualitative estimate of functions not covered by the other. Thus the PAd test is not influenced by a low platelet count, whereas the BT test is uninfluenced by variations in the PCV. It is, however, important to recognise that normal results with both tests do not exclude the possibility of mild abnormalities of the platelet aggregation tests, consequently the aggregation tests must be carried out if there is clinical evidence of an unusual bleeding tendency.

The Place of a Plasma Factor in Platelet Adhesiveness

It is now established that in von Willebrand's disease the PAd is reduced, but other platelet function tests are normal [30]. We have found that mild variations in the other platelet function tests do not influence the PAd results, only gross variations of platelet aggregation tests, such as occur in the syndromes described in this paper, are accompanied by reduction in the platelet adhesiveness. This particular reduction may well be due to failure of that part of the platelet loss during the passage over the glass beads that depends on the formation of platelet aggregates on the beads. In von Willebrand's disease it is known that there is a plasma factor that influences the BT test since infusion of normal plasma will correct the BT [19, 26] and the low PAd result (15). It is possible that it is variation in this plasma factor that produces the variation in platelet adhesiveness encountered in normal persons, rather than in the platelets themselves as shown in the various other platelet function tests.

It appears that a reduced PAd represents either a low PAd plasma factor or a platelet defect; a PAd within the normal limits indicates that the platelet aggregate is normally formed (but a mild platelet functional abnormality is not excluded) and finally a high PAd probably represents an elevated PAd plasma factor.

Acknowledgements: We thank Mr. ARIS SERSTRAS for the statistical analysis of our results, Miss M. SHORROCK for technical assistance and the Illustration Department of the United Manchester Hospitals.

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Acta haemat 46 14-23 (1971)

Hemolytic Anemia Associated with Decreased Concentration of Reduced Glutathione in Red Cells

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Abstract Three cases of hemolytic anemia with decreased concentration of reduced glutathione in red cells are described They all have had signs of hyperhemolysis from early infancy and 2 exchange-transfusions had to be performed in one of them In 2 of them stomatocytes are present in their blood smears and in one of them high sodium and low potassium in the erythrocytes is observed Family studies reveal several apparently healthy members with the same deficiency and presenting evidence of mild to moderate hyperhemolysis. Genetic transmission was dominant autosomal The phenotypic expression seems to differ greatly In 1 case who presented with gallstones at the age of 19 years splenectomy beneficially influenced the intensity of the hemolytic process.

Key Words
Glutathione of erythrocytes
Enzyme erythropathy
Haemolytic anaemia
Heinz bodies
Hereditary anaemia
Stomatocytes

Glutathione is present in high concentration in human erythrocytes The main part of this tripeptide is kept in the reduced form whereas the lesser part is found as oxidized glutathione Although there is an increased susceptibility to hemolysis in reduced glutathione deficient red cells, the significance of the low concentration of reduced glutathione itself as a possible factor in the hemolytic process is largely unknown We have recently investigated 3 unrelated families in which a hereditary diminution of red cell reduced glutathione has been repeatedly demonstrated

Methods

Routine laboratory investigations were carried out by standard conventional methods. The methods for the determinations of glycolytic enzymes in hemolysates

have been described elsewhere [4]. Reduced glutathione was assayed according to the method of STEVENSON [20] and its stability was determined by the method of BEUTLER *et al.* [2]. Methemoglobin was measured by the standard method of EVELYN and MALLOY [6]. Red-cell sodium and potassium were estimated by the methods of ZAKROWSKY and OSKI [17, 24].

Case Reports

Case 1 is a girl, born 1950. She had persistent anemia and jaundice during the first 2 years of her life but remained in good health since 1952. There is no history of taking drugs except occasional aspirin. 1969 gallstones were removed and at the same time splenectomy was performed. She is currently well. Results of pre- and postoperative hematological as well as family studies are summarised in table I. Prior to the splenectomy the patient had hemolytic anemia. The reduced glutathione was diminished whereas other enzyme activities (G-6-PD, 6-phosphogluconate dehydrogenase, glutathione reductase, and hexokinase) were within normal limits. Postoperatively, the level of reduced glutathione remains low but there is no evidence of hemolysis. Her mother and her younger sister have fairly well compensated hemolytic anemia and they too have diminished reduced glutathione but normal activities of other erythrocyte enzymes. After incubation with acetylphenylhydrazine the reduced glutathione in the red cells of the patient and her relatives appears to be stable. However, they differ from normal erythrocytes by the formation of Heinz bodies. Methemoglobin levels are within normal limits. No striking abnormalities are present in the blood smears, the direct antiglobulin test is negative and osmotic fragility and autohemolysis tests, as well as liver function tests are normal in all of them. The genealogical tree (fig. 1) shows that her father and one other younger sister are not affected. Unfortunately we had no opportunity to investigate the rest of the family.

Table I. Results of investigation from case 1 and her relatives

	Patient		Mother	Younger sister	Normal values
	preoperative	postoperative			
Hemoglobin, g%	8.8	14.5	13.1	12.3	
Reticulocytes, %	16.0	0.8	5.9	8.4	
Serum bilirubin, mg%	2.5	1.5	0.8	1.0	
Haptoglobin, mg%	25.0	65.0	35.0	34.0	40–190
GSH, mg/100 ml eryth	42.0	46.0	46.0	42.0	50–95
GSH after ACPH mg/100 ml eryth		47.0	45.0	35.0	>25.0
Heinz body test		+	+	+ +	negative
Methemoglobin, %		0.45	0.5	0	0–0.5

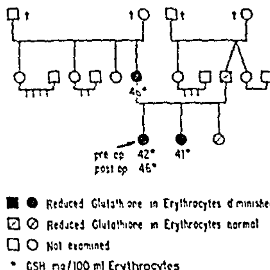


Fig. 1. Pedigree of the family of case 1.

Case 2 is a 9-year-old girl. She was jaundiced after birth and required 2 exchange transfusion. At 11 months old she was readmitted because of anemia, jaundice and splenomegaly. Laboratory investigations showed Hb 11 g%, retic. 20%, eryth 3.3 mill/mm³. Serum bilirubin 2.1 mg% (mainly indirect). Direct antiglobulin test negative. Incubated osmotic fragility increased. Autohemolysis 6.8% and with added glucose 4.2% after 48 h incubation at 37 °C. Red cell life span 14 days and ⁵¹Cr half life of red cells 8 days. Bone marrow showed increased erythropoiesis (83% erythroblasts). The findings were in favour of a congenital non spherocytic hemolytic anemia. However, after reviewing the hematological investigations and in particular the serial blood smears during the follow up periods from 1962 to 1969, a diagnosis of congenital hemolytic anemia with stomatocytosis, high sodium, low potassium, and low level of reduced glutathione in erythrocytes was made. Relevant findings in 1969 are shown in table II. In the blood picture typical stomatocytes are seen and the majority of the red cells are macrocytes (fig. 2). Family study (fig. 3) reveals that her mother has slightly diminished reduced glutathione in the red cells but without evidence of hemolysis. No stomatocytosis has been detected in other members of her family.

Case 3, a 4-month-old male infant, was found to have a hemolytic anemia with stomatocytosis. The hemoglobin was 9 g%, reticulocytes 5.5%. There were about 25% stomatocytes (fig. 4), 3% nucleated red cells, and some basophilic stippling in the erythrocytes. The child had in addition multiple deformities (congenital heart defect, micrognathy, kyphoscoliosis, klinodactyly, syndactyly, and congenital dislocation of the hips) and died soon after admission of respiratory failure. In this family (fig. 5) low level of reduced glutathione has been found in his father, brother, paternal aunt and 2 paternal cousins. They are otherwise in good health and hematologically, no abnormalities are present.



Fig. 2 Blood picture of case 2.

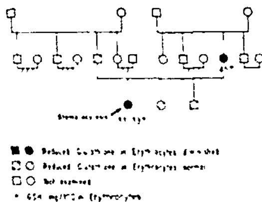


Fig. 3 Pedigree of the family of case 2.

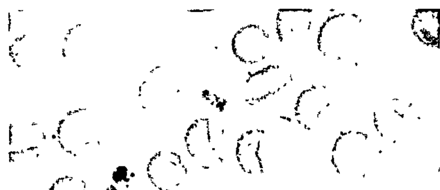


Table II. Results of investigation from case 2

Clinical		
Pallor, slight jaundice, splenomegaly (4 cm below costal margin), hepatomegaly (1 cm below costal margin)		
Hematological		Normal values
Hemoglobin, g%	9.8	
Reticulocyte count, %	3.3	
Hematocrit, %	35	
MCV, μm^3	128	
MCH, pg	35.8	
MCHC, %	29	
Erythrocytes diameter, μ	7.62–8.22	
Stomatocytes, %	33.4	
Red cell sodium, mEq/l	65.2	(13.5±2.3)
Red cell potassium, mEq/l	33.6	(95.5±4.0)
Serum Fe, μg%	79	
Haptoglobin, mg%	32.5	
Serum bilirubin (mainly indirect), mg%	2.2	
Direct antiglobulin test	negative	
Incubated osmotic fragility	increased	
Autohemolysis moderately increased and only slightly reduced by the addition of glucose in 48 h at 37 C		
Autohemolysis at 5 C	normal	
Reduced glutathione, mg%	33–39	(50–95)
Glucose-6-phosphate-dehydrogenase, IU	39	(12–22)
Glutathionreductase, IU	22	(7–14)
6-phosphogluconate dehydrogenase, IU	34	(10–16)
Hexokinase, IU	7.4	(0.8–2.0)
Phosphoglyceromutase, IU	117	(20–68)
Pyruvate kinase, IU	106	(24–56)
Hemoglobin F, %	[illegible].5	(0.25–0.75)
Hemoglobin A_2, %	1.5	(1.5–3.0)

Discussion

Hereditary hemolytic disorder associated with reduced glutathione deficiency has been reported in 6 different families in Holland [16, 19], Germany [12, 23], France [3] and USA [15] (table III). Combined with stomatocytosis it has been described in one case by MILLER *et al.* in

Table III. Hemolytic anemia associated with GSH-deficiency

Authors	Number of cases	Severity of anemia	Glutathione		
			% of normal	Stability	HB[1]
Oort *et al.* [16]	5	mild	10	–	+
Löhr *et al.* [12]	1	moderate	10	unstable	+
	father	–	50	unstable	+
	2 brothers	–	80	unstable	+
Waller and Gerok [23]	1	severe	50	unstable	+
	1 son	–	60	unstable	+
Boivin *et al.* [3]	1	moderate	10	–	+
	1	moderate	10	–	+
Mohler *et al.* [15]	1	mild	< 10	unstable	+
Case 1	1	moderate	70	stable	+
	mother	mild	70	stable	+
	1 sister	mild	70	stable	+

[1] HB = Heinz body test

Table IV. Hemolytic anemia associated with stomatocytosis and low concentration of GSH

Authors	Number of cases	Severity of anemia	Glutathione		
			% of normal	Stability	HB[1]
Miller *et al.* [14]	1	moderate	60	unstable	+
Case 2	1	moderate	45		
Case 3	1	moderate	?		

[1] HB = Heinz body test

USA [14] (table IV). Our cases are the first to be documented in Swiss families.

Aspirin has been claimed to accentuate hemolysis in G-6-PD deficiency [21] and in reduced glutathione deficient red cells, hemolysis increased after ingestion of fava beans or primaquine [16, 19]. In case 1 and her relatives, no oxidant drugs or their metabolites other than occa-

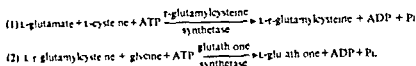


Fig. 6. Synthesis of glutathione

sional aspirin to case 1 have been administered and clear cut relationship between drug ingestion and hemolysis could not be established. Compared with those described by OORT *et al.* [16], LÖHR *et al.* [12], BOIVIN *et al.* [3] and MOHLER *et al.* [15] the level of reduced glutathione in our cases and in those of WALLER *et al.* [23] is somewhat higher than 50% of normal. Yet the degree of anemia reported seems to be inversely proportional to the level of reduced glutathione (table III). JACOB and JANDL [8] have observed that the glutathione content of red cells can be lowered to 10% when they are treated with N-ethylmaleimide without affecting their metabolism *in vitro* and their survival time *in vivo*. It appears therefore that other unknown factor or factors may be involved in the process of hemolysis in glutathione deficiency. Furthermore glutathione abnormalities has been described in various hematological disorders [1, 7, 10, 16, 18, 22, 25] and it is possible that low concentration of glutathione is not the primary defect, but secondary to certain metabolic disorders of the red cells. However, our observations provide further evidence that this disorder is a heterogeneous one.

It has been shown by different authors [3, 15, 16] that in glutathione deficient erythrocytes the glutathione synthesis is disturbed. It could be demonstrated that the red blood cells were lacking in glutathione-synthetase but not in r-glutamylcysteine synthetase (fig. 6). In case 1 and her relatives, the glutathionereducing system in the abnormal red cells is intact. This is supported by the fact that the activity of glutathione reductase is either normal or increased. The normal glutathione stability suggests that abnormal destruction or degradation is not involved. Therefore, it is most probable that the primary disturbance is one of glutathione synthesis.

The mode of inheritance has been claimed to follow an autosomal recessive pattern [12, 16, 23]. Our investigations, on the contrary, suggest that the reduced glutathione deficiency is inherited as a dominant trait. As in the case described by BOIVIN *et al.* [3] splenectomy seems to have had beneficial effect on our case 1.

In case 2 and 3 the metabolic abnormality in the red-cell membrane plays an important rôle in the process of hemolysis. There is no definite correlation between reduced glutathione deficiency and stomatocytosis. Not all reduced glutathione deficiency cases have stomatocytosis or *vice versa*, although stomatocytosis has been observed in other hemolytic disorders [5, 9, 11, 13, 17, 24]. The significance of decreased concentration of reduced glutathione here is unknown. Case 2 appears to be similar to the one described by MILLER *et al.* [14] with the exception that the erythrocytes in our patient are not susceptible to cold hemolysis *in vitro*. The value of splenectomy in stomatocytosis is open to speculation. The cases described by MILLER, ZARKOWSKY and OSKI [14, 17, 24] have shown clinical improvement after removal of spleen inspite of persistent abnormal hemolysis and alter red cell abnormalities.

Acknowledgement. Our thanks are due to Dr. R. LANDOLT in Chur for referring the Patient case 1 to us.

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Acta haemat. 46: 24–35 (1971)

Enzyme Cytochemistry of the Energy Metabolism in the Cells of Normal Human Bone Marrow

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Key Words
Bone marrow
Energy metabolism
Enzyme cytochemistry
Gel film technique

Abstract. Using a gel film technique 11 key enzymes of the energy metabolism were histochemically demonstrated in the bone marrow cells of healthy persons or patients suffering from non-haematological disorders. The enzyme activity was assessed by a semiquantitative scoring method. A reduction of all enzymatic activities could be seen in the erythropoiesis during maturation. No histochemically visible change of enzymatic activity was observed with increasing differentiation of the granulopoiesis in the LDH, GAPDH, MDH, ME, GLUDH, GDH and HK. However, the enzymatic activity increased in the PGLUM and G6PDH whereas the SDH and GPOX activity diminished with the maturation of the granulopoiesis. It was concluded that the gel film technique may reveal increasing or diminishing alterations in the enzymatic pattern in diseases of the bone marrow.

It is difficult to draw conclusions from experiments on bone marrow homogenates because of the heterogeneity of the material. This is also true for diseases of the bone marrow, for example pernicious anemia because there are always left different parts of the normal cell population which come under consideration [5, 7]. So far the enzymatic pattern of the individual cells can only be ascertained by the use of histochemical techniques, such techniques which can also reveal changes in the enzymatic cell pattern in the case of bone marrow diseases.

However, before carrying out histochemical experiments on haematologically diseased cells the enzymatic pattern of the different cell series in haematologically healthy people must be known. This paper will therefore deal with the histochemical demonstration of eleven key enzymes of the energy metabolism in normal, human bone marrow cells.

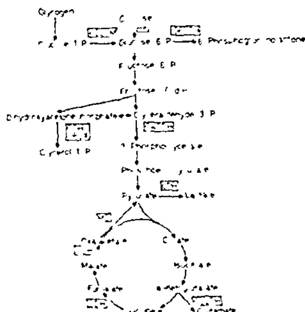


Fig. 1. Scheme of the energy linked metabolism with glycolysis, hexosemonophosphate shunt and citric acid cycle. The histochemically demonstrated enzymes are drawn in

Materials and Methods

The gel film technique newly developed by PETTE and BRANDT [10] was used. The principle of this method depends on the reduction of the tetrazolium salt to the insoluble coloured formazan. Fifteen marrow samples were taken from healthy persons or patients suffering from non-haematological diseases. The cytochemically demonstrated enzymes are shown in Figure 1.

The following abbreviations are used: G6PDH = glucose-6-P-dehydrogenase, HK = hexokinase, PGLUM = phosphoglucomutase, GAPDH = glyceraldehyde-3-P-dehydrogenase, LDH = lactate dehydrogenase, SDH = succinate dehydrogenase, MDH = malate dehydrogenase, ME = malic enzyme, GLUDH = glutamate dehydrogenase, GDH = glycerol-3-P-dehydrogenase, GPOX = glycerol-3-P-oxidase.

After the preparation the smears were air-dried and fixed in pure cold acetone (+4 C) [1, 8] for exactly 60 sec. The incubation of the smears took place 30 min after the bone marrow aspiration. All the slides were stained at the same time. Up to this time the slides had been stored at +4 C.

Details of substrate concentrations used in the applied technique may be found in the papers of PETTE and BRANDT [10] and SIGEL and PETTE [11]. Assays for

Table I. Scheme of the semiquantitative scoring procedure

Mode of reaction	Reaction class
No reaction	0
Faint circumscript reaction	1
Weak diffuse reaction or moderate circumscript reaction (< 50% of the cytoplasm)	2
Moderate diffuse reaction or intense circumscript reaction (≥ 50% of the cytoplasm)	3
Intense diffuse reaction	4
Strongly positive diffuse reaction	5

GLUDH, MDH, GDH, GPOX and MI were incubated for 3 h, all the others for 1 h. When using multiple step reactions (HK, PGI UM) we did not add G6PDH, the enzyme catalyzing the indicator reaction, but relied upon the cells endogeneous enzyme. Phenacine methosulfate (PMS) was used as a redox mediator. Assays without substrate served as controls.

Following the incubation, the slides were fixed again in 4% formalin and were counterstained with nuclear fast red for 5 min [11]. Subsequently they were examined with a high power oil immersion lens without coverslide.

Evaluation was semiquantitative, using the data in table I. Twenty five cells of each developmental stage were evaluated consecutively and the strength of reaction judged according to table I. The index of each cell type was obtained by multiplying the sum of the cells counted for each reaction class with the grade of the reaction class.

Results

In contrast to PETTE and BRANDAU [10] and SIGEL and PETTE [13] who studied enzymes in tissue sections, we worked on entire cells although with damaged membranes. We therefore increased the substrate concentrations up to 4 times the amount reported in the original papers. However, despite the necessity for membrane permeation, there was proportionality of incubation time and substrate turnover even at the original substrate concentrations given by PETTE, BRANDAU and SIGEL. Therefore these concentrations were subsequently used.

The substrate free assays used as controls never exhibited any reaction. Incubation in a nitrogen atmosphere did not increase the colour intensity.

Incubation times varying from 15 min to 5 h were checked for all enzymes. The time after which no further formazan precipitation was

Table II. Semiquantitative estimation of the different erythropoietic stages (M ± σ)

Erythroblasts	Early	Intermediate	Late
HK	–	–	–
PGLUM	69 ± 2	51 ± 1	49 ± 1
G6PDH	58 ± 2	46 ± 3	39 ± 2
GAPDH	67 ± 2	52 ± 2	44 ± 2
LDH	66 ± 3	49 ± 2	44 ± 2
SDH	73 ± 1	56 ± 1	47 ± 1
MDH	64 ± 3	51 ± 3	42 ± 2
ME	60 ± 5	47 ± 2	41 ± 3
GLUDH	–	–	–
CPOX	60 ± 3	40 ± 3	32 ± 3
GDH	58 ± 3	47 ± 4	34 ± 5

noted even when the incubation was of a longer duration, was used for the assays.

The formazan precipitation appeared diffuse and light to dark blue. Only rarely did the microcrystals aggregate to form single coarse granules which appeared superimposed upon the diffuse stain. No certainty was obtained about demonstration of enzyme activity in nuclei. Occasional circumscript areas of nuclear staining may be attributable to cytoplasm overlying the nucleus.

The erythroblasts were judged as early, intermediate and late, since the counterstain used did not always permit exact classification. The results of the activity count are shown in table II [17]. A significant decrease of all enzyme activities from proerythroblasts to orthochromatic normoblasts is evident (fig. 2a–c). HK- and GLUDH activities could not be demonstrated with certainty in normal erythropoietic precursors. In various disease entities, however, an increase of these enzyme activities could be shown. No activity could be demonstrated in erythrocytes with this method. Some dehydrogenases were in evidence at the site of the *tetrazolium reductases* when PMS was omitted from the assays (fig. 2d) [17].

Results concerning leukopoietic stages are summarized in table III [15]. The intensity of the reaction was almost the same for each enzyme within a given developmental stage (fig. 3). Some of the enzymes detected in the granulocytic series *did not exhibit histochemical differences between*



Fig. 2. The diminishing enzyme activity in the erythropoiesis shown by means of the SDH. *a* Pronormoblast. *b* Intermediate normoblasts. *c* Late normoblast. *d* LDH activity in the erythrocytes at the site of the NADH tetrazolium reductase (the platelets show an intense reaction).

Table III Semiquantitative scored index of the leucopoiesis (M ± σ)

	1	2	3
HK	60 ± 4	60 ± 4	60 ± 4
PGLUM	76 ± 3	82 ± 4	100 ± 0
G6PDH	80 ± 2	86 ± 2	100 ± 0
GAPDH	75 ± 0	75 ± 0	75 ± 0
LDH	75 ± 0	75 ± 0	75 ± 0
SDH	48 ± 2	58 ± 2	53 ± 0
MDH	75 ± 0	75 ± 0	75 ± 0
ME	60 ± 4	60 ± 4	63 ± 4
GLUDH	50 ± 0	50 ± 0	50 ± 0
GPOX	72 ± 3	70 ± 4	55 ± 4
GDH	75 ± 0	75 ± 0	75 ± 0

1 = Promyelocytes
2 = Myelocytes
3 = Metamyelocytes and polymorphonuclear neutrophils



Fig. 3 GAPDH in 9 multinucleated granulocytes

the stages from promyelocytes to multinucleated granulocytes. These include LDH, GDH, MDH, ME, GAPDH, GLUDH, and HK.

Another group in which the activity increases as the maturation proceeds comprises SDH and GPOX. Highest activities were observed

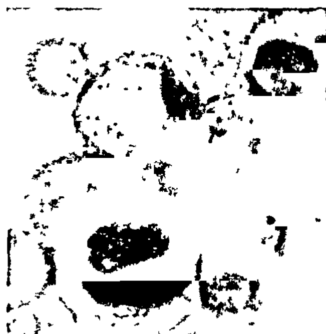


Fig 4 Demonstration of PGLUM in the granulopoietic cell series Lighter reaction in the promyelocyte (upper part of the picture) than in the myelocyte (lower part of the picture) and the more differentiated stages

in promyelocytes However the heterogeneity of the promyelocytic group is reflected, in variations of colour intensity in these youngest cells of normal leukopoiesis There were also cells with reaction grade 3 and others with reaction grade 2, which is the same intensity as in the more advanced phases

A 3rd group comprising G6PDH and PGLUM showed decreasing activity with increasing maturation (fig 4) This increase in activity is already discernible in some of the promyelocytes From the metamyelocytes on, the cell population is homogeneous again when judged by its enzyme activities

The eosinophilic series is readily recognizable with the staining procedure we used (fig 6a) The enzyme content is below that of the corresponding developmental stages of the neutrophils since the eosinophilic granules occupy only a part of the cell cytoplasm Only for G6PDH and PGLUM were higher activities detected in eosinophils than in neutrophils Megakaryocytes feature an enzyme activity pattern of greater intensity

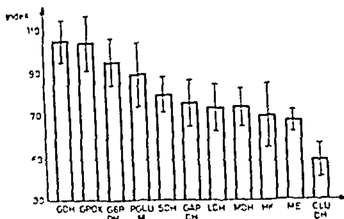


Fig 5 Semiquantitative estimation of the reaction grade in the megakaryocytes (mean and twofold standard deviation)

than the other cell series (fig 5) There were considerable differences in enzyme activities between individual giant cells

Discussion

The histochemical demonstration of dehydrogenases is related to their catalytic activity However, topically correct formazan precipitation can only be expected if the endogeneous tetrazolium reductase, which serves as hydrogen acceptor, is present in sufficient quantity at the localization of the enzyme According to our observations, the NADH- and NADPH-dependent tetrazolium reductases are demonstrable in the granulopoietic series with small amounts and uneven distribution only PMS as oxido-redox mediator overcomes the heterogeneous distribution of the diaphorases as the limiting step in the chain of reactions This warrants maximal enzymatic activity at the site of reaction which represent the site of the enzyme localization The histochemical detection of dehydrogenases using the soluble assay mixture entails the leakage of soluble extra-mitochondrial enzyme activities into the incubation medium Using cryostat sections of rat liver PETTE and BRANDAU [10] were able to demonstrate that 50 % of total LDH activity penetrates into the incubation

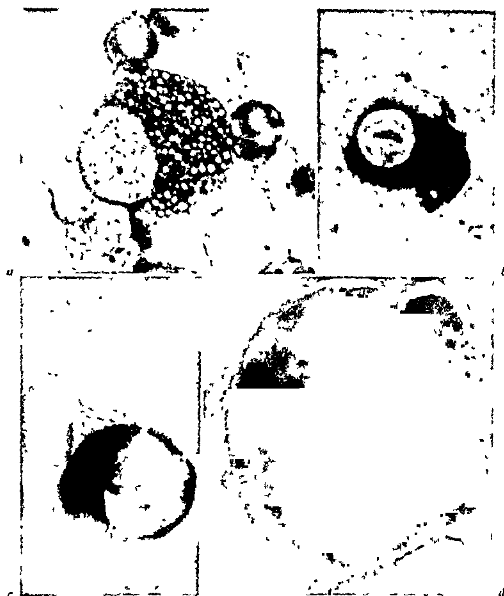


Fig 6 *a* LDH in an eosinophilic myelocyte *b* GPOX in a plasma cell *c* SDH in a promyelocyte *d* SDH in a megakaryocyte

medium during 15 min of incubation These artifacts are prevented by using the gel layer technique, since the diffusion of the relatively large protein molecules is prevented mechanically by the structure of the gel An augmentation of formazan precipitation following incubation in a



Fig. 7. SDH activity in a reticulum cell

nitrogen atmosphere as compared to incubation in air can not be expected, since the hydrogen uptake of nitro blue tetrazolium occurs at a lower level of the redox potential than that of oxygen. For this reason, ALTMANN [2] detected no differences between aerobic and anaerobic incubation in a quantitative comparison of nitro blue tetrazolium reduced by G6PDH.

According to SCHMIDT *et al.* [12], the enzyme diffusion through the damaged cell membrane is primarily dependent upon the concentration gradient and the molecular weight. During perfusion of rat liver under hypoxic conditions, these authors obtained a slow release of G6PDH into the perfusion medium which was still insignificant after 1 h. We used the cells endogeneous G6PDH in reactions where G6PDH was needed as the indicator enzyme. G6PDH has to be considered as the limiting member in the chain of assay reactions for PGLUM which is present with high activities particularly in leucocytes.

One major obstacle remains in the use of a fixative. Acetone accomplishes denaturation of protein by breaking up hydrogen bonds [3]. MELNICK [8] when comparing a number of fixatives found that pure cold acetone yields best results in a compromise between enzyme inactivation and maintenance of cell morphology. Apart from the maintenance of cellular appearance, the acetone fixation is advantageous, since

the extraction of lipoids from the cells reduces formazan precipitation at enzyme free locations through absoption at lipid containing structures [4, 6, 9, 15, 16]

Contrary to our expectations, we did not find a granular distribution of the formazan pattern for mitochondrial enzymes In the case of GLUDH a fine and diffuse formazan precipitation was present even in leukopoietic and thrombopoietic cells SDH and GPOX featured a localized confluent cloudy precipitation which is particularly prominent in plasma cells, promyelocytes, megakaryocytes (fig 6b–d) and reticulum cells (fig 7) In these cases, we assume that the pattern corresponds to mitochondrial activities, even though the mitochondrials are not shown This kind of intramitochondrial enzyme demonstration might be explained as a consequence of a diffusion of reduced PMS into the vicinity, since the local amount of tetrazolium is limited due to the high local enzyme activities

The subjective judgment upon which the semiquantitative assessment of reaction intensity is based, is subject to *many imponderables* This disadvantage can be minimized, if only results obtained under identical conditions are related to each other, or if quantitative evaluations with the use of cytophotometric methods are employed, such as were recently reported by STUART *et al* [14]

Acknowledgement The valuable technical assistance of Mrs Ch OESTERREICH is acknowledged

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Acta haemat. 46: 36–44 (1971)

Pentose-Phosphate Pathway of Leucocytes

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Abstract. The pentose phosphate pathway of leucocytes tested with the transketolase reaction (TKA) was markedly and strongly reduced in chronic lymphatic leucemia. A slighter degree of pathway inhibition was noted in diabetics, alcoholics and patients with azotemia or myeloma. The plasma of patients receiving diphenylhydantoin was found to inhibit the pentose phosphate pathway of the leucocytes.

Key Words
Diphenylhydantoin
Leukocyte metabolism
Lymphatic leukemia
Pentose phosphate shunt
Transketolase

Leucocytes have a predominantly aerobic glycolytic metabolism [1–3]. Pentose-phosphate shunt activity, measured in terms of transketolase activity, is probably 100–200 times that of red cells [17, 21]. In an earlier study the authors showed that the transketolase activity (TKA) of leucocytes *in a* healthy human body remains relatively constant for a given number of leukocytes at different hours during day and night, and in the same person from one day to another [21]. As shown earlier, in connection with numerous enzymological studies [4–7], leucocytes are well suited to studies of pentose phosphate pathway.

Material and Methods

The series consisted of 199 persons. A detailed distribution and characteristics are given in tables I and III. The group numbers of table I are the same as those of figure 1.

1. Controls (104). All were healthy subjects in good condition. 36 were men (age 50.0 ± 16.6 years, body weight 75.3 ± 16.9 kg, Hb 13.7 ± 1.6 g/100 ml) and 65 women (*36.9 ± 21.7, 64.8 ± 10.2, 12.9 ± 0.9*).

[1] Grant from the National Research Council for Medical Sciences, Finland.

Table I Characteristics of the material studied, the group numbers are the same as in figure 1

No	Group	n	Age, years ±SD	Weight, kg ±SD	Hb, g 100 ml ±SD
1	controls	104	41.5 ± 20.9	70.9 ± 15.3	13.3 ± 1.3
2	rheumatoid arthritis	40	54.4 ± 12.2	64.0 ± 7.2	12.4 ± 1.5
3	diabetes mellitus	12	61.9 ± 17.7	73.5 ± 18.1	12.0 ± 2.0
4	chronic alcoholism	10	45.5 ± 13.0	82.5 ± 17.6	11.2 ± 3.2
5	bronchial asthma	9	44.3 ± 20.2	68.2 ± 8.4	12.8 ± 0.3
6	azotemia	7	64.4 ± 11.0	56.4 ± 11.0	9.9 ± 3.2
7	multiple myeloma	5	69.6 ± 22.0	67.8 ± 15.3	11.1 ± 1.4

Table II The white cell TKA in 5 test subjects treated with diphenylhydantoin

Patient	TKA units 10^{9} leucocytes	
	plasma dilution	NaCl dilution
1	2.8	9.0
2	3.4	5.0
3	2.3	6.8
4	3.8	8.5
5	5.2	15.3

In plasma dilution the white cells were diluted after separation with autogenous plasma. In the second test the autogenous white cells were diluted in physiological saline solution. The plasma produced a 2- to 3-fold inhibition.

2. *Rheumatoid arthritis (40).* The disease was diagnosed on the basis of visible joint changes, roentgenological changes, rheumatoid serology, and elevated ESR (54 ± 27 mm/h). The patients had a history of chronic rheumatoid arthritis averaging 11 years.

3. *Diabetes mellitus (12).* The diabetes had been diagnosed in adult age, and the patients were in hospital in order to achieve equilibrium with the disease. Some had taken insulin and other an oral antidiabetic drug. A number of the patients had had diabetic complications.

4. *Chronic alcoholism (10).* The patients had been admitted for alcohol intoxication and re-examined during convalescence. Special neurological examination revealed signs of neuritis in the extremities of all patients. All had a history of several years of alcoholism.

5. *Bronchial asthma (9).* All patients had been admitted for an acute attack of asthma. Specimens were taken during the course of an attack. All had 5–15% eosinophils in peripheral blood.

Table III White cell TKA of patients with blood diseases

Patient No	Diagnosis	Sex	Age years	Weight kg	Hb, g%	Leuc /mm³	TKA, units/10⁹ leuc
1	CLL	f	80	70	11 4	41,000	2 9
2	CLL	f	75	63	11 3	21,700	1 1
3	CLL	m	67	67	12 6	8 600	1 2
4	CLL	m	65	45	8 8	24 800	1 8
5	CLL	m	63	65	9 4	10 400	0 7
6	CLL	f	64	50	12 2	18 500	1 6
7	CLL	m	23	59	8 3	20 000	1 4
8	CLL	f	78	57	11 4	34 600	1 4
9	CLL	m	59	68	12 5	35,900	1 5
10	CLL	f	65	57	6 6	24,200	3 0
11	CML	f	57	48	11 7	10,100	6 5
12	CML	f	38	46	13 0	12,000	10 6
13	CML	m	77	62	6 0	120 000	6 9
14	CML	m	67	65	11 5	60 000	17 1
15	CML	f	50	81	6 1	100 000	3 3
16	ALL	f	19	40	9 6	60 000	6 4
17	AA	m	47	80	7 0	1,100	1 3

CLL = Chronic lymphatic leucemia, CML = chronic myeloid leucemia ALL = acute lymphoblastic leucemia, and AA = aplastic anemia NB the heavily reduced TKA values in CLL and AA

6 *Azotemia (7)* All were patients examined at the hospital with markedly elevated serum creatinine values and reduced excretion of phenol red

7 *Multiple myeloma (5)* All these patients had been examined under hospital conditions Their diagnosis was based on examination of the bone marrow and electrophoresis and on radiography of the bone system

A number of patients with blood diseases primarily leucemias are assembled in table III All had been examined under hospital conditions, and the diagnosis was based on the examination of peripheral blood bone marrow and, in some cases, lymph glands

The isolation of leucocytes from fresh blood samples has been described earlier [21] At first the isolated leucocyte masses were diluted with autogenous plasma [21] Later examinations of certain drug takers especially those of diphenylhydantoin revealed that plasma inhibited the white cell transketolase activity Maximum enzyme activity was obtained when the cells were suspended in physiological saline Subsequently only physiological saline dilution was used and it appeared to produce maximum enzyme activity in all cases

Another point of the method on which we digressed from our earlier practice [21] was that the parallel red cell sample was excluded The reason was that the transketolase activity of a parallel red cell sample has often been zero or a negligible fraction of the enzyme unit

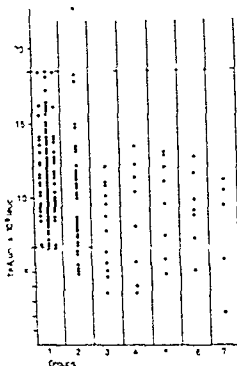


Fig. 1. The TKA of the leukocytes. (1) controls (mean ± SD 11.6 ± 2.9 units/10^9 leuc.) (2) rheumatoid arthritis (10.7 ± 4.0 units) (3) diabetes mellitus (5.0 ± 2.8 units) (4) chronic alcoholism (8.7 ± 3.3 units) (5) bronchial asthma (9.8 ± 3.0 units) (6) azotemia (9.3 ± 2.5 units) and (7) multiple myeloma (6.6 ± 3.4 units).

Otherwise the treatment of the samples and enzyme determinations took place as previously described [21]. The white cell TKA was indicated in units: μmole sed-heptulose-7-phosphate/10^9 leucocytes/30 min, 37 °C, pH 7.4.

The statistical treatment of the results was carried out by computer (at the Institute for Applied Mathematics, University of Turku). The results are presented in table I–III and figure 1.

Results

Figure 1 and its caption present the specified results for the groups examined. The TKA of the control group was 10.1 ± 2.5 units in men (36) and 12.4 ± 2.8 units in women (45). The difference is not significant.

The white cell TKA in chronic rheumatoid arthritis and bronchial asthma did not significantly differ from that of the controls

The TKA in diabetes (t = 4 012), chronic alcoholism (t = 2 955) azotemia (t = 2 012) and myeloma (t = 3 704) was significantly lower than that of the controls The confidence limit was 5%

Table III reveals that the white cell TKA in chronic lymphatic leucemia (CLL), in every case examined, was distinctly below the range of variation in the controls In chronic myeloid leucemia (CML) and acute lymphatic leucemia (ALL) the TKA was below the lower limit of the normal variation (6 6 units/10^8 leuc) in 3 cases out of 6, most of the values were close to the lower limit, and they can, therefore, not be definitely considered as reduced values In one case of aplastic anemia the patient's white cell TKA had fallen steeply, to the same level as in chronic lymphatic leucemia

Table II gives 5 patients treated with diphenylhydantoin The TKA was determined on white cells suspended in autogenous plasma which contained diphenylhydantoin and/or its metabolites, and again on autogenous white cells suspended in physiological saline not containing the drugs TKA determined on saline solution showed an activity 2-3 times that of TKA determined on plasma dilution containing drug metabolites An aqueous solution containing 0 0, 2 0 and 4 0 mg diphenylhydantoin per 100 ml was then prepared with suitable test leucocytes This experiment showed that the addition of diphenylhydantoin *in vitro* did not inhibit the transketolase reaction of the white cells (0 mg 10 7 units/10^8 leuc , 2 mg 10 6 units, and 4 mg 10 7 units)

Discussion

The principal findings from this study of white cell TKA were as follows In chronic lymphatic leucemia, TKA may be as much as 10 times less than in the controls Inactivation was also noted in the diabetics, alcoholics patients with azotemia or with myeloma In patients treated with diphenylhydantoin the plasma factor acts as TKA inhibitor, whereas diphenylhydantoin added *in vitro* did not inhibit the TKA reaction

It has been recorded that glucose-6-PD and 6-phosphogluconate dehydrogenase activity in leucemic lymphocytes is reduced [12 22] and that lymphocytes in leucemia contain excess glycogen [14] A lowered glucose tolerance has been noted in patients with chronic lymphatic leucemia [19] The significantly diminished quantities of $^{14}CO_2$ evolved from glucose 1-^{14}C by the leucemic lymphocytes compared with its normal relative, is evidence

that this neoplastic cell metabolizes proportionately less sugar through the pentose phosphate pathway [9, 10]. The present results are parallel to, and consistent with the above observations. The white cell TKA was heavily reduced in CLL in all patients examined to date, but not so distinctly and definitely in the other leucemias. In one case of aplastic anemia following the use of analgesics the white cell TKA showed the leucemic level. Further studies are required to show whether this is a general characteristic in aplastic anemia. If the answer is in the affirmative, heavily reduced TKA values in white cells will not constitute binding evidence in the enzymological differential diagnosis of CLL. For later studies in our laboratory, the TKA of isolated healthy lymphocytes equals TKA levels of other white cells [unpublished data].

Diabetics' white cells often behave in TKA determinations in the same way as those of patients with chronic lymphatic leucemia, even though the change in the former is not so distinct as in the leucemics. This is similar to the general clinical observation, that both diabetics and lymphoproliferative disease patients have a predilection to bacterial, mycotic and granulomatous infections. It has also been observed that a new immunosuppressive drug ICI 47,776 has an inhibitory action on the phosphorylation of mitochondria and on ATP generation while it also interferes with the use of ATP in glycolysis [11]. It might be assumed that disturbed sugar metabolism in immunologic white cells is perhaps in some way associated with the immunologic activity of these cells. Reduced TKA in diabetic white cells (total cells) suggests the same conclusion as the results of BRODY and MERKER on lymphocytes [10]. Even though the TKA of patients with bronchial asthma (fig. 1) is identical with that of the controls, the white cell TKA in some dermal allergies seems to fall remarkably [unpublished finding].

In the majority of the examined alcoholics the pentose phosphate pathway of the red cells is inactivated [20]. They seem to have the same predilection in white cells also, although the change is not particularly marked. Systemic examinations also have shown that alcohol has mainly an inhibitory action on glucose metabolism [18].

According to the present study, a predisposition towards the inactivation of pentose phosphate pathway seems also to exist in myeloma and azotemia. Patients with azotemia have long been known to have a reduced immunologic response [15].

Diphenylhydantoin has been suspected to induce a variety of hypersensitivity reactions [23], including lymphadenopathy, leucopenia, thrombocytopenia, pancytopenia, dermatitides, erythema multiforme [25], serum

sicknesses [8], lupus erythematosus [24] and lymphocytic thyroiditis [16]. Diphenylhydantoin has induced blast-like transformation in patients' lymphocytes [13].

The present study revealed that the plasma of a patient treated with diphenylhydantoin inhibited the transketolase reaction of the white cells. A similar though not so pronounced phenomenon has been noted in the red cell TKA [unpublished observation]. Since diphenylhydantoin added to white cell dilution *in vitro* failed to produce an inhibition, it may be assumed that it is produced by drug metabolite(s) formed in human organism, or that binding with plasma proteins changes the enzyme-inhibiting capacity of diphenylhydantoin. Since inhibition of pentose phosphate pathway is involved here, it should be recalled that diphenylhydantoin has been reported to have an inhibitory effect on glucose metabolism in some studies [26–33]. The ultimate causes of this inhibitory action have not been established. It is possible that the present finding, inhibition of the transketolase reaction by plasma of patients receiving diphenylhydantoin, might be associated with the diabetogenic action of this drug.

Acknowledgement. Our thanks are due for technical help to M. PUKKA, M.Sc. (Biochemistry).

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Acta haemat 46 45-49 (1971)

Effect of RNA Extracted from a Neoplastic Cell Line on Lymphocyte Cultures

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Abstract RNA extracted from a cell line GH7, derived from a patient with lymphosarcoma, was added in varying amounts to lymphocyte cultures from the patient and from normal blood donors An inhibitory effect of high doses on all cultures and a stimulating effect of low doses on the patient's cultures were observed Several hypotheses are proposed to explain the results obtained and the mechanism of action of the RNA added

Key Words
Cell culture
Lymphocytes
Lymphosarcoma
Neoplastic cells
RNA

It is well known that bacterial [4] vegetable [8] and mammalian cells [11] can undergo morphological and functional transformation under the effect of nucleic acids It has also been shown that the addition of ribonucleic acid (RNA) to PHA-stimulated lymphocyte cultures leads to inhibition of blastic transformation [1 2 9] and that there is an inhibition of nuclear RNA synthesis in Ehrlich ascites cells which have been incubated with homologous or heterologous RNA [5]

The object of our experiments was to determine the effect of RNA extracted from a cell line, GH7, derived from a patient (RDL) with lymphosarcoma [10] on *in vitro* short term cultures of peripheral lymphocytes obtained both from the patient RDL and from normal blood donors

Methods

RNA was extracted at 65 C by the method of Travnicek *et al* [12] using a mixture of 90% phenol and 0.1M sodium [illegible] buffer pH 6 and dissolved in a

small amount of buffered saline solution The quantity of nucleic acid obtained was measured by the orcinol reaction [3]

The method used for lymphocyte culture is based on that reported by Moorhead *et al* [7] Heparinized blood from the patient and normal donors were allowed to sediment in glass tubes at room temperature for 1-2 h The supernatant plasmas were removed, pooled and samples of 4 ml were pipetted into glass bottles containing 5.5 ml of TC 199 medium (GIBCO) The basic suspension was varied by the addition of RNA in concentrations ranging from 100 to 1,000 μg culture The patient's and normal lymphocyte cultures were subdivided into the following groups (1) controls (without RNA), (2) low doses of RNA (100-160 μg/culture), (3) intermediate doses of RNA (300-700 μg culture) and (4) high doses of RNA (800-1,000 μg/culture)

After the 3rd, 7th and 14th day of incubation, slide preparations of the cultures were stained with May Grünwald Giemsa, periodic acid Schiff (PAS) and acridine orange 1,000 cells of the lymphocytic population were examined, and per cent blast cells determined

Results

The normal lymphocyte cultures showed no significant differences in blast percentage compared to the respective control group, when lower and intermediate doses of RNA were added However, lower values were obtained with higher doses

The patient's lymphocyte cultures presented a significantly different behavior as compared to the control group ($P = 0.01$) The highest initial percentage of blast transformation (42%), was observed with the intermediate doses of RNA, although there was a marked decrease on the 7th and 14th day The effects of lower and higher doses of RNA appeared mainly on the 14th day of culture. In the former, there was a stimulation of the transformation, the percentage of blast cells observed being 68%. On the contrary, in the latter, there was inhibition, the percentage of blast cells being 11%. Both values differ significantly ($P<0.01$) from the controls of the same day 38%. The results are summarized in table I

Samples taken from the patient's cultures with the addition of low doses of RNA showed on the 7th and 14th day of incubation giant multinucleated cells, with slight cytoplasmic basophilia, and in some cases, presence of granulations in the nuclei and cytoplasm

Smears stained with PAS showed positive cells only in the groups to which low doses of RNA had been added The other groups, including controls, were practically negative It must be pointed out that the cell line GH7, from which the RNA had been extracted, was PAS positive

Table 1 RNA added to peripheral lymphocyte cultures. Blast cells (% ± SD) after 3, 7 and 14 days of incubation

RNA added	3rd day P (1)	3rd day N (2)	7th day P	7th day N	14th day P	14th day N
	%	%	%	%	%	%
Control without RNA	10 ± 1.4	25 ± 0.3	25 ± 0.3	13 ± 0.8	39 ± 0.8	15 ± 1.1
Low doses 100–160 μg	27 ± 1.8	22 ± 0.4	46 ± 0.5	20 ± 1.2	68 ± 0.8	15 ± 0.3
Intermediate doses 300–700 μg	42 ± 2.3	25 ± 1	22 ± 0.3	11 ± 0.3	23 ± 1.2	10 ± 0.4
High doses 800–1000 μg	22 ± 0.2	15 ± 0.5	12 ± 1.4	10 ± 1.2	11 ± 0.3	0 ± 0.6

(1) Patient's lymphocyte cultures. (2) Normal blood donors' lymphocyte cultures.

As for acridine orange stains, it was found that the percentage of positive cells increased with the addition of progressive doses of RNA in cultures from the patient and normal subjects.

Discussion

On the basis of these results, it can be concluded that: (1) the lower doses of RNA (100–160 μg) possess a stimulating effect on the patient's lymphocyte cultures, which is specially marked at the 14th day of incubation; (2) the intermediate doses of RNA (300–700 μg) have an initial stimulating effect on the patient's cultures, which disappears on the 7th day of incubation; and (3) the higher doses of RNA (800–1000 μg) seem to possess an inhibitory effect on both types of cultures, which again is more marked on the 14th day of incubation.

The differences observed between varying doses of RNA and their mechanism of action is not easy to explain. RIGBY *et al.* [9] observed suppression of human lymphocyte transformation in PHA stimulated cultures when they added 0.3 mg or more of yeast RNA. The same effect was observed with polyuridylic acid when PHA was not added to the cultures. With smaller amounts of RNA there was an apparent stimulation of the transformation. They correlated their findings of suppressed

orrhages. Patient No 7 was a 60-year-old male, who died for diffuse bronchopulmonitis, resistant to antibiotic treatment, and final collapse

Patient No 8 also died following sepsis, resistant to antibiotic treatment Patient No 9 died incidentally following a car accident a year after the beginning of the disease.

Patient No 10 died following an unrestrainable gastric hemorrhage Patient No 14 died for cerebral hemorrhage after a slow downhill course

Other 2 patients (cases 15 and 16) having a hyperplastic bone marrow, died respectively for a pulmonary infarction during a drug induced jaundice and for sepsis

The other patients had a favorable course, following treatment with androgens, except patient No 18, in whom erythroleukemia developed

The following investigations which are important to establish the diagnosis of aplastic anemia were carried out blood cell counts, leukocyte differential counts, reticulocyte count, bone marrow biopsy, ferrokinetics, alkaline phosphatase in neutrophil granulocytes, and alkali resistant I hemoglobin level Some of these data are reported in tables I and II

The criteria on which diagnosis was based, were the presence of pancytopenia, reticulocytopenia, low red cell iron turnover rate, high level of alkaline phosphatase in neutrophil granulocytes and bone biopsy Patients No 1–14 had an aplastic or severely hypoplastic bone marrow, patients No 15–18 had a hyperplastic bone marrow and for them a diagnosis of ineffective erythropoiesis was established Details of these data are not given here, because these features of the disease were already discussed thoroughly by previous authors [25]

Only some data concerning the ferrokinetics of 2 patients are reported in table II Patient No 1 having an aplastic bone marrow had a very low red cell iron turnover rate, which increased considerably after 6 months of treatment with testosterone Patient No 18, affected by refractory anemia with hyperplastic bone marrow, displayed peculiar aspects of ferrokinetics a low ^{59}Fe $T_{1/2}$ which corresponds to a high plasma iron turnover, but a very low uptake of ^{59}Fe by the erythrocytes, the uptake by the liver is very high, suggesting that the iron available in the plasma is rapidly bound by liver tissue

Liver Function

Liver function has been studied by determination of serum transaminases, bilirubinemia, bromosulphonftalein retention, mucoproteins and serum γ-globulins At least 7 of our patients had increased levels of serum transaminases. This occurred in patient No 1 in occasion of 2 episodes of hepatitis with a parallel increase of bilirubinemia, and was likely due to serum hepatitis, as the patient had previously received many blood transfusions At that time Australia antigen test was not yet carried out as a routine test

Other 2 patients (cases 14 and 17) had increases of transaminases accompanied by slight increase of bilirubinemia. In another patient (case 16) a temporary slight increase of serum transaminases occurred with a concomitant marked increase of serum bilirubin. In these patients it remained uncertain whether liver involvement was due to serum hepatitis or androgen toxicity

In another patient (case 15) there was an increase in bilirubinemia not accompanied by an increase in transaminases and this was probably the only case in which a definite liver damage due to androgen treatment occurred.

Other 6 patients showed occasionally very slight increases in serum bilirubin, while serum transaminases were normal.

Lastly, in 2 patients (cases 2 and 3) serum transaminases were above normal with variable values in different times, but serum bilirubin was always normal. With regard to the possible relation between high transaminase levels and testosterone treatment, it is to be noted that in these patients serum transaminases were high before starting the androgen treatment. However, in some phases of the disease in which androgen treatment was temporarily withdrawn there was occasionally a fall in serum transaminase levels.

Glucose Metabolism

Of our 18 patients, 3 were affected by diabetes (cases 15, 16 and 17). Other 6 patients developed hyperglycemia briefly after starting glucocorticoid treatment. Patients No. 1-3 and 6 developed hyperglycemia and glycosuria just after beginning glucocorticoid treatment and hyperglycemia persisted until glucocorticoids were withdrawn. These patients were not known to be diabetic from the history. Other 2 patients (cases 7 and 8) became hyperglycemic a few weeks after the beginning of glucocorticoid treatment. In other 2 patients there was occasionally mild hyperglycemia and glycosuria (cases 14 and 18). In the remaining patients hyperglycemia was never observed.

Immunologic Conditions

These were studied evaluating the lymphocyte count, skin hypersensitivity reactions, immunoglobulin levels and the presence of plasma cells in the bone marrow.

The differential blood cell counts showed a high percent of lymphocytes particularly in the first group of patients (cases 1-12) with typical pancytopenia. This was due to a marked granulocytopenia, however also the absolute number of lymphocytes was slightly decreased.

The delayed hypersensitivity reactions (tuberculin, Candida, Streptomyces) tested in a few patients were negative.

Plasma cells were always present in the bone marrow. Gamma globulin values were in general normal in the majority of the patients. However, they were definitely low in patient No 2. Other 2 patients (cases 6 and 11) had γ-globulin values lower than normal or at the lower limits of the normal. On the contrary, there are patients having γ-globulin levels constantly elevated, independently from temporarily associated infectious processes: patient No 7, who also had an elevated fibrinogen level and a tendency to gel formation by the serum at low room and elevated temperatures, and patients No 8 and No 14. No definite correlations with depressed or excited immune mechanisms could be established in aplastic anemia.

Treatment

The treatment has been in the majority of the cases the traditional association androgens-glucocorticoids [1, 20, 22, 25]. A hundred milligram of testosterone

Table I

Case No	Age years	Blood cell counts per mm³ erythrocytes × 10⁶	leukocytes × 10³	platelets × 10³	Lymphocytes %	Reticulocytes ‰	Transaminases	Bilirubinaemia
1	13	2.0–4.0	1.5–2.5	10–50	40–85	0–40	+ + +[1]	+ + +
2	60	1.7–3.0	1.0–1.5	15–50	25–70	0–30	+ + +	normal
3	27	1.5–3.0	1.5–3.0	10–30	50–80	1	+ +	normal
4	33	0.85–3.0	0.8–3.0	2–25	36–74	7–11	normal	normal
5	21	1.0–3.0	1.8–2.7	12	96–100	5–10	normal	normal
6	26	1.5–3.0	3.2–3.4	15	86–96	26	normal	normal
7	60	1.6–3.8	1.1–2.2	2–26	60	0	normal	normal
8	49	2.5–3.5	4.0–5.4	2–16	30–63	9	normal	+
9	34	1.4–3.7	1.8–3.0	5–18	85	2.7–4.1	normal	normal
10	56	2.7–3.2	2.0–3.0	8–40	50–85	5	normal	normal
11	40	1.0–2.6	2.0–3.8	6–22	65	5–11	normal	normal
12	65	2.0–4.6	1.0–2.3	96–122	60	20	normal	+ +
13	34	2.0–4.0	1.3–5.8	200	32–53	10	+	+
14	77	1.1–3.3	2.5–5.0	106–184	30–50	13–26	+ + +	+ +
15	73	2.0–2.5	3.0–4.0	164	25	0–40	normal	+ + +
16	60	2.0–3.5	1.5–4.0	81–119	40	22	+	+ + +
17	59	1.64–3.7	1.4–3.3	83–110	35	0.5	+	+
18	59	1.9–2.3	0.7–11.5	110	35	16–25	normal	+

[1] The signs + + +, + +, + refer to different extents of increase of biological values, occurring during the course of the disease, compared to the normal

propionate and 10–40 mg of prednisone were given daily *per os*. However, in 5 patients, at present under treatment, glucocorticoids were no longer given in the last 12 months and only androgen treatment was continued.

Patient No 1 deserves particular mention. He was affected by a severe form of the disease. He received about 80 blood units in 6 months, and after having been treated with 100 mg of testosterone propionate and 40 mg of prednisone daily for 4 months, was still in danger of life. Owing to the appearance of severe cortisonism symptoms, like hyperglycemia, 'striae cutaneae', and finally an enterorrhagia, which required the transfusion of 5 units of blood in a few hours to safe the life of the patient, prednisone treatment was withdrawn and the patient continued to be treated with testosterone propionate only. In a few weeks not only the enterorrhagia stopped completely and the hyperglycemia and the 'striae cutaneae' disappeared, but also a definite hematological improvement occurred. Reticulocytosis appeared for the first time, a good hemoglobin level was maintained without need of other blood transfusions, leukocyte and platelet counts improved, the patient is now at home living an almost normal life.

Mucoproteins mg%	Glycemia	Total serum proteins g%	Serum globulins, g%			
			α_1	α_2	β	γ
5-6	++	4.8-7.2	0.24-0.28	0.40-0.84	0.70-0.97	0.67-1.31
3-6	+++	5.5-5.9	0.19-0.32	0.25-0.45	0.57-0.94	0.47-0.75
5	++	5.9-6.3	0.29-0.33	0.45-0.74	0.64-1.06	0.90-1.09
9	normal	7.1	0.21	0.70	0.85	1.14
9-12.5	normal	6.7	0.27	0.89	0.65	1.53
4.7	+	5.5-5.9	0.20-0.22	0.60-0.45	0.67-0.79	0.44-0.96
4.1	+	7.1-7.8	0.25-0.35	0.66-0.90	0.95-1.11	1.85-2.51
3.5	+	6.7-7.8	0.23-0.32	0.60-0.68	0.95-0.99	1.87-1.67
5.4-7.4	normal	7.8-6.7	0.45-0.37	0.95-0.77	1.01-1.09	0.94-0.67
4	normal	7.1	0.29	0.64	0.83	1.03
4.4	normal	5.9	0.13	0.30	0.56	0.63
6.8-12.8	normal	5.9-6.3	0.26-0.25	0.37-0.46	1.19-0.80	1.35-1.50
3.3	normal	5.2-6.7	0.21-0.22	0.35-0.50	0.76-0.95	0.78-1.20
3	+	7.1-8.6	0.16-0.25	0.33-0.43	0.55-0.90	2.15-3.97
4.6	+++	4.5-7.5	0.11-0.18	0.25-0.53	0.56-1.0	0.81-2.3
4	+++	6.3-6.9	0.26	0.41-0.49	0.77-0.82	1.15-1.27
4.8-6	+++	7.1	0.16	0.27	0.70	1.33
5.9-6.2	++	5.5-6.7	0.20-0.22	0.31-0.36	0.75-0.83	0.52-0.92

When 2 values for a patient are reported these correspond to the higher and lower values found during the course of the disease

In other 4 patients, glucocorticoids have now been withdrawn with a definite improvement in the hematological situation and a disappearance of hyperglycemia and glycosuria which persisted also with very low prednisone dosage (5-10 mg daily).

Discussion

A few points might deserve to be briefly discussed on the basis of the data here presented and the data published in the literature

Concerning liver function in patients affected by aplastic anemia, the same unknown factors which cause aplastic anemia in some patients might also be toxic for the liver It has also to be considered that cases of liver disfunction, due to preexisting hepatitis have been reported in aplastic anemia [17]

Table II Ferrokinetics

	Patient No 1		Patient No 18
	before testosterone treatment	after 6 months of testosterone treatment	
^{59}Fe $T_{1/2}$, min	245	120	60
Plasma iron turnover rate (mg/24 h/100 ml)	40 2	67 4	136
Uptake of ^{59}Fe into red corpuscles after 7 days, %[1]	21 6	85 1	13.2
Uptake of ^{59}Fe over			
sacrum	absent	high	low
liver	early and high	low	high
spleen	high and late	low	low

[1] The uptake of iron into the red corpuscles is expressed as a proportion of the amount injected

Hepatitis following blood transfusions appears to be responsible for the majority of cases of liver involvement In dubious cases, in which the clinical symptoms are not pathognomonic, the possibility of an uncommon, i e anicteric, variety of hepatitis must be considered The presence of Au antigen in the blood of these patients might help to establish this diagnosis

With regard to liver toxicity following androgen therapy, in our series only in 1 case treated with methyltestosterone, androgen toxicity was considered as certain This patient presented the signs of cholostatic icterus marked serum hyperbilirubinemia, elevated serum alkaline phosphatase, while serum transaminase levels were within normal values

Concerning glucose metabolism, 3 of our patients were affected by diabetes, other 6 patients developed hyperglycemia and glycosuria following slight doses of glucocorticoids This led us to consider the possibility that in these patients there was a particular sensitivity to glucocorticoid treatment

The immunological conditions of the patients were studied particularly evaluating the serum γ-globulin values A patient had hypo-γ-globulinemia. A case of hypo-γ-globulinemia associated with aplastic anemia was described by DREYFUS *et al* [6] A few patients had hyper-γ-globulinemia, in the absence of chronic hepatitis infections or other pathological conditions generally associated with hyper-γ-globulinemia In no

case a monoclonal peak of γ-globulins was found. The majority of our patients had normal γ-globulin values.

Several reports have been recently published on the use of androgen therapy [1, 20, 22, 25]. In spite of some controversial data [20] on the long term effect, there is no doubt on the satisfactory results of this treatment in the majority of the patients. A mechanism of action of androgens seems to be an increased secretion of erythropoietin but other mechanisms of action probably play a role in stimulating erythropoiesis [7, 9, 10]. The side effects of this therapy, liver toxicity and virilization in females, justify temporary withdrawal of treatment followed by a trial with another androgen only in case of icterus. Concerning virilization, experiments are now being carried out on new 5β H-steroid metabolites devoided of virilizing effects, which will probably be used currently in clinical practice in a near future [10, 11].

Also a small dose of glucocorticoids is generally associated to androgen therapy [1, 23, 25]. The clinical results obtained in our patients do not seem to justify this treatment. No worsening of symptoms was noted in any patient and, on the contrary, severe side effects, like enterorrhagia, hyperglycemia, 'striae cutaneae', osteoporosis and moon face returned to the normal after withdrawal of glucocorticoids. Also the hematological conditions improved in some patients. Other authors [5, 22] suggest that glucocorticoids are unuseful and even might be dangerous in the treatment of aplastic anemia. This, however, appears to be a controversial point considering the opinions of different authors [1, 23, 25, 29].

Under theoretical point of view glucocorticoids treatment would not appear to be useful. Aplastic anemia syndrome is probably due to many different etiologic factors, and might also have differing pathogenetic aspects. Chloramphenicol is a frequent cause of aplastic and refractory anemia [2, 24], and at least in this case some considerations might be made on the pathogenesis, since the mechanism of action of chloramphenicol has been studied at a molecular level [8, 28]. Chloramphenicol inhibits protein synthesis by interacting with ribosomes. In mammals mitochondrial protein synthesis is specifically inhibited [18, 27].

Glucocorticoid action is not so definitely known, except perhaps on lymphatic tissue [12, 13, 15, 16, 19, 26]; however, it is known that glucocorticoids have, in general, a catabolic effect opposite to that of androgens, and inhibit protein synthesis. Furthermore, it has recently been shown that glucocorticoids inhibit erythropoiesis in normal rats [7, 10]. Therefore the use of glucocorticoids in the therapy of aplastic anemia

Acta haemat 46 60-63 (1971)

Modifications de la résistance osmotique des hématies au cours des maladies hémolytiques par auto-anticorps[1]

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Abstract. In patients with haemolytic diseases due to auto-antibodies the erythrocytes show an increased fragility. The authors suggest hypothetical explanations for these changes.

Key Words
Autoantibodies
Erythrocyte membrane
Haemolytic anaemias
Osmotic fragility

L'étude de la fragilité osmotique des hématies permet, avec certaines hypothèses, l'approche des propriétés rhéologiques de la membrane érythrocytaire [6]. C'est ainsi que par la comparaison des courbes d'hémolyse lente par dialyse et d'hémolyse rapide (technique de Dacie), il est possible de définir les paramètres d'élasticité et de viscosité de la membrane globulaire [8]. Le but de ce travail est l'étude des paramètres d'hémolyse dans le cas des maladies hémolytiques à auto-anticorps, où la présence d'une molécule étrangère fixée sur la membrane, peut, peut-être, en modifier certaines propriétés mécaniques.

Méthode

Mode de détection des maladies hémolytiques. Le sang prélevé sur citrate est séparé en 2 parties: plasma et culot d'hématies. Le culot est lavé 6 fois en eau physiologique à 37 °C et d'autre part à 4 °C, et est resuspendu à 5% en eau physiologique.

[1] Ce travail a été réalisé avec l'aide de la DRME, section Biologie. Contrat No 70-34-034-00-480-75-01.

Le test direct de COOMBS [1] s'effectue à l'aide de 2 serums anti-γ-globuline polyvalent differents Si le test est positif, on utilise alors le sérum anti-γ-G, anti-γ-M et anti complement, de telle sorte que nous classons les anemies en 5 categories γ-M+C, avec agglutinines froides dans le serum γ-M+C sans agglutinines froides, complement, γ-G et mixte [5–7]

Mesure des fragilites osmotiques Afin de pouvoir envisager l'etude des paramètres rheologiques nous avons pour chaque cas étudie les parametres de fragilite obtenus par hémolyse lente (dialyse) et par hémolyse rapide.

Les courbes de fragilité par dialyse [3] sont obtenues automatiquement à l'aide d'un appareil automatique appele «fragiligraph», alors que l'hemolyse rapide est obtenue par la technique classique des tubes d'ionicite decroissante [9] Sur chacune de ces courbes et afin d'envisager un traitement statistique des resultats, nous n'avons considéré que 3 points qui sont le début le milieu et la fin d'hemolyse.

Résultats

Nous avons compare statistiquement, pour chacun des 6 seuils d'hémolyse la population des valeurs normales (55 cas) à la population des maladies hémolytiques (12 cas ou 11 cas) (tab I)

Le test utilisé est celui de Student Fisher Apres calcul des parametres statistiques nous pouvons conclure avec un risque de 5‰ que les 2 populations sont differents c'est à-dire que la presence d'un auto-anti-

Tableau I Modifications des paramètres d'hémolyse au cours des anemies hemolytiques

	Début d'hémolyse	Milieu d'hémolyse	Fin d'hemolyse	Nombre de cas étudies
Hemolyse lente (fragiligraph)				
valeurs normales	4 83±0 33	3 66±0 40	1 74+0,50	55
malad e hémolytique	5 68±0 99	4 27±0 48	2,10±0 79	12
signification statistique (test de Student Fisher)	t=15 45	t=9 15	t=4 02	significatif à 5‰ si t≥1,96
Hémolyse rapide (Dac e)				
valeurs normales	5 77±0 40	4 48±0,25	3 72±0,37	55
maladie hémolytique	7 85±2,66	6 19±1 60	4 11±2,30	11
signification statistique	t=27 55	t=28 60	t=5,30	significatif à 5‰ si t≥1,96

corps sur l'hématie provoque une modification des paramètres de résistance globulaire.

Conclusion

Parmi les hypothèses qui peuvent justifier de telles modifications, citons (1) la présence d'une *molécule* étrangère (anticorps) fixée sur la membrane, et (2) des variations éventuelles de la composition chimique de la membrane (lipides en particulier). Dans cette voie, les travaux de COOPER [2] semblent montrer que le taux en cholestérol et en phospholipides de la membrane est abaissé au cours des anémies hémolytiques (environ 10%). C'est d'ailleurs ce que prouve une expérience chez le rat qui, après l'injection d'anticorps incomplets, provoque une baisse de 20 à 30% du cholestérol et des phospholipides. Cependant, JAFFE et GOTTFRIED [4] ont décrit récemment une famille de 8 membres atteints de maladie hémolytique et dont le cholestérol et les phospholipides totaux étaient normaux, mais où l'on pouvait noter une augmentation de 25% des lécithines.

Résumé

L'étude des paramètres d'hémolyse d'hématies de malades atteints de maladie hémolytique par auto-anticorps montre une fragilisation de celles-ci. Les auteurs envisagent alors des hypothèses pouvant justifier ces modifications.

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Libri

H. Stobbe: Hämatologischer Atlas. Zytomorphologie, Zytochemie und Funktion der Zellen von Blut und Knochenmark. Darstellung hämatologisch wichtiger Krankheitsbilder sowie Methodik der morphologischen Blut- und Knochenmarksuntersuchung. 3., völlig neu bearbeitete Auflage. Akademie-Verlag, Berlin 1970. 246 Cpl., 190 fig., 19 tab. Preis: DM 96.–.

Die Neuauflage des hämatologischen Atlas von Stobbe wurde mit grosser Sorgfalt überarbeitet. Neben den zahlreichen informativen Farbmikrophotos enthält der Band Phasenkontrastaufnahmen, einige elektronenmikroskopische Bilder sowie zahlreiche, übersichtliche Tabellen und Schemata. Die verschiedenen hämatologischen Krankheitsbilder werden in einem meist recht ausführlichen Textteil entsprechend dem neuesten Stand des Wissens eingeführt. In einem Anhang finden sich detaillierte Methoden, die für die zytologische Beurteilung hämatologischer Erkrankungen von besonderer Bedeutung sind. Auch ein Verzeichnis der wichtigsten hämatologischen Standardwerke und Zeitschriften fehlt nicht. Dieses qualitativ hervorragende Übersichtswerk dient nicht nur dem Fachhämatologen, sondern auch jedem praktisch tätigen Arzt. Die didaktisch geschickte Darstellung wird aber auch vom Laborpersonal ausserordentlich geschätzt.

E. A. Beck, *Bern*

Progress in Medical Genetics, vol. 7. A. G. Steinberg and A. G. Bearn (eds.). Grune & Stratton, New York 1970, XI+243 pp., 0000.

The seventh yearly volume of Progress in Medical Genetics once again consists of an invaluable series of articles on a variety of topics, some of general genetic and others specifically of medical genetic interest.

Barbara R. Migeon and B. Childs report on Hybridization of mammalian somatic cells, D. Y. Hsia on Phenylketonuria and its variants, S. Ruddy and K. F. Austen on Inherited abnormalities of the complement system in Man, M. K. Fagerhol and C. Laurell on the Pi system-inherited variants of serum α_1 antitrypsin, M. D. Milne on Genetic aspects of renal diseases, H. E. Sutton on The haptoglobins, and W. C. Parker on Some legal aspects of genetic counseling.

Each article includes extensive literature lists and there are authors and subject indexes. The table of contents does not simply consist of the titles of the seven articles as in former volumes but has been expanded to provide detailed subtitles. This makes it easy to have an early overview of the extent of the treatment of the main topic and to look up specific details. The only criticism which the reviewer wishes to raise concerns the continued unusual mode of type setting of the references, which results in unnecessary efforts in locking for the names of authors cited.

C. Stern, *Berkeley, Calif.*

Acta haemat 46 65–73 (1971)

Induction of Remission in Myeloblastic and Monoblastic Leukaemia in Adults with Rubidomycin

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Abstract 20 adults with acute myeloblastic, mono myeloblastic or monoblastic leukaemia have been treated with Rubidomycin. 6 Patients achieved complete remission and 2 partial remission Smaller dosages of Rubidomycin were used than in previous series and there was a lower incidence of irreversible marrow hypoplasia Myocardial toxicity was found to be much commoner in the older patients

Key Words
Leukaemia
Rubidomycin
Therapy of acute leukaemia

Rubidomycin is now regarded as the most effective single drug for the induction of remission in acute leukaemia in adults [14] Earlier reports from specialised centres have indicated that remissions might be expected in about 25% of adult patients [4, 5, 10] although a remission rate of 50% has been reported [2] However, the powerful effects of this drug on the bone marrow and its undoubted cardiotoxicity have limited its more general use The disappointing remission rates with other drug regimes justify its wider use and it is clear that good results can be obtained using smaller dosages with less risk of fatal marrow aplasia [15] We report here the results of treatment of 20 adults with acute myeloblastic, mono myeloblastic and monoblastic leukaemia

Patients

The 20 patients were aged from 17 to 77 years and formed a consecutive series admitted to the Nottingham hospitals during the period October 1968 to June 1970 The details of these patients are shown in table I All were diagnosed as suffering from acute myeloblastic mono-myeloblastic and monoblastic leukaemia

Table I

Age years	Sex	Diagnosis	Previous drugs	Drugs given with Rubidomycin	Total Dosage of Rubidomycin mg	mg/kg	Alopecia	Cardiotoxicity
64	M	AMML	S, 6MP	S	360	5.0	+	–
17	M	AML	S, V, 6MP	S	300	5.0	–	–
20	F	AML	S, 6MP	S	200	4.0	–	–
16	M	AML	–	S	140	3.0	–	–
55	M	AMML	S, V, 6MP	S	320	5.0	–	+
77	F	AMonL	–	S	275	2.5	–	–
33	M	AML	S, 6MP	S	1,200	15.0	–	+
44	F	AML	S, 6MP	S	125	2.0	–	–
63	F	AML	–	–	340	5.5	–	–
49	M	AML	S	S	780	13.0	–	+
43	F	AML	S	S	510	10.0	–	–
72	F	AMonL	–	–	540	9.0	–	+
62	M	AML	–	–	660	10.5	+	+
72	M	AML	–	S	360	6.0	–	+
65	M	AMML	–	S	616	8.0	–	+
71	F	AMonL		–	570	9.5	+	+
50	M	AML	–	S, V	435	7.5	–	–
37	M	AML	–	S, V, 6MP	670	11.0	–	–
17	M	AML	S	S	420	5.5	–	–
[illegible]	F	AML			360	3.0	+	+

= failure AML = acute myeloblastic leukaemia S = steroids
= complete remission AMML = acute mono-myeloblastic leukaemia 6MP = 6-mercap
= partial remission AMonL = acute monoblastic leukaemia V = Vincri

8 of the patients had previously received other treatment but all were in relapse when treated initially with Rubidomycin. 1 had attained a previous remission with other treatment.

Method of Treatment

Rubidomycin was given in dosages of 0.5–2 mg/kg body weight intravenously with a fast flowing infusion into an arm vein. At the onset 2.0 mg/kg was given as an initial dose, but later this was reduced to 1.0 mg/kg. Injections were repeated at 3-day intervals until the total white blood count fell to less than 1,000/µl. This was usually achieved within 10 days and thereafter the dosages were reduced according to the appearances of the peripheral blood film and/or the bone marrow findings. When less than 1% of blast cells remained in the peripheral blood smear

tine bone marrow examinations were carried out and further injections of Rubidomycin given in an attempt to eradicate blast cell activity from the marrow In a number of patients this was prevented by the associated depression of normal erytho-, leuko- and thrombopoiesis in the bone marrow After full treatment with Rubidomycin there was usually a phase of marrow aplasia or hypoplasia lasting from 10 to 30 days during which no chemotherapeutic agents were given If remission was not obtained at this stage further injections of Rubidomycin were given These were not usually effective in inducing late remissions, although some partial remissions were obtained in this way When complete remission occurred the patients were maintained with oral 6-mercaptopurine

During the hypoplastic phase of the treatment the patients were barrier nursed in isolation cubicles Naseptin cream was applied to the anterior nares routinely and the patients were given prophylactic antibiotics or chemotherapy in the form of ampicillin, cephaloridine or sulphamethoxazole and trimethoprim (Septrin®) Blood transfusions were given as required and platelet rich blood administered in severe thrombocytopenic states Leukocyte infusions were not given Electrocardiograms (ECG) were carried out twice weekly on all patients before and during therapy We found that many patients became depressed during the protracted stay in hospital and the majority were allowed home for short spells of a few days at suitable periods during their therapy No patients were informed of the diagnosis

Results

The clinical details and results of treatment are summarised in table I and figure 1

General Haematological Effects

Of the 20 patients treated 3 obtained complete remission with treatment with Rubidomycin alone and 3 with steroid therapy *in addition* The criteria for complete remission were those outlined by HOLLAND *et al* [6] and included a normal peripheral blood count, a normal bone marrow with active erythropoiesis, leukopoiesis and thrombocytopoiesis, with less than 5% of blast cells present, and no abnormalities on physical examination

In all the patients except 1 (case 9) the administration of Rubidomycin resulted in a distinct reduction in the numbers of circulating blast cells and also in the total leukocyte count. The most dramatic effect was seen in case 8 where there was a precipitous fall in the total white cell count of 135,000, comprising 98% blast cells, to a count of 2,800 within 5 days of a single injection of 2 mg/kg Rubidomycin Unfortunately, this patient did not wish to continue with a full course of treatment and the full impact of Rubidomycin on the disease process could not be as-

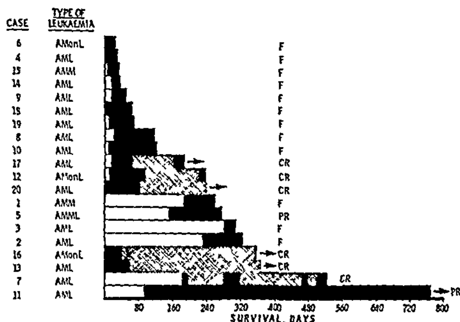


Fig 1 ■ Duration of treatment with Rubidomycin, ▨ Complete remission following rubidomycin Results F = failed, CR = complete remission, PR = partial remission

sessed Only 1 patient (case 9) showed no response in the whole series and the total white blood count rose from 8,000 to 120,000 in 15 days whilst 5 5 mg/kg Rubidomycin was being administered The period of hypoplasia, defined as a leukopenia of $<1{,}500/\mu l$ after Rubidomycin varied usually from 1 to 3 weeks, but in 1 patient (case 13) the total white blood count remained persistently below 1,000 for 8 weeks before complete remission ensued and it is of interest that this remission has been maintained with 6-mercaptopurine for a year without further treatment

One patient was considered to have died from marrow aplasia as a direct result of therapy but it was often difficult to assess the degree of marrow failure on examinations of the peripheral blood and bone marrow On occasions when bone marrow biopsy indicated complete aplasia bone marrow trephine examinations showed remaining islets of haemopoietic tissue

Complete Remissions

In the 6 patients in whom complete haematological remission was obtained the period of remission has been from 6 weeks to a year and 3

are still alive and well Usually the patients have been maintained in remission on 6 mercaptopurine in dosages varying from 50 mg on alternate days to 100 mg daily

In 1 patient 3 remissions were obtained with repeated courses of Rubidomycin In this patient the value of Rubidomycin in controlling incapacitating bone pain was clearly demonstrated. Case 7, a 33-year-old farm worker, was admitted with a 6-month history of increasing tiredness loss of 6 kg weight and anorexia. He had had tuberculous glands excised from the neck at the age of 12 but had had no previous significant past history On examination he was pale with petechial haemorrhages over his ankles the spleen was palpable 3 cm below the left costal margin but there was no lymphadenopathy Investigations showed haemoglobin 8.2 g/100 ml total nucleated cell count 5 000/μl 13% bands, 29% neutrophils, 19% myelocytes 3% eosinophils 24% lymphocytes 1% monocytes, 11% nucleated red cells. Bone marrow examination showed acute myeloblastic leukaemia. He was treated with blood transfusion prednisolone and 6-mercaptopurine and remained in fair health for about 5 months when his haemoglobin fell to 7 6 g/100 ml and he began to suffer bone pains. X rays showed multiple lesions in lumbar vertebrae the left femur and the iliac crests. Complete haematological remission was obtained with 3 mg/kg Rubidomycin with a gratifying reduction in bone pain Further relapse and an exacerbation of the bone pain occurred another 6 months later and further remission was achieved with 4 mg/kg Rubidomycin. A third and transient remission was obtained again 4 months later with 4 mg/kg and the patient eventually d ed 18 months after the initial diagnosis At post mortem (Dr P G SMITH) the bone lesions were shown to be due to leukaemic deposits

In 1 patient (case 13) the induction of remission after 10.5 mg/kg Rubidomycin was preceded by a probable period of haemolysis At this stage, when the haemoglobin level was only 4 1 g/100 ml there was a reticulocytosis of 18% and very many spherocytes were present the Coomb s test was negative Steroid therapy was increased to 30 mg prednisolone/day and the reticulocyte count fell and there was a corresponding prompt rise in haemoglobin level At the same time the total white cell count rose to a maximum of 51 000/μl with 58% neutrophils, 17% metamyelocytes, 13% myelocytes 5% lymphocytes and 7% monocytes. Subsequently this leukemoid peripheral blood picture reverted to normal

Failure to Achieve Remission

14 of the 20 patients failed to attain complete haematological remission Of these 2 were considered to achieve partial remission. One patient (case 11) has now survived more than 2 years with a peripheral blood count often entirely normal Short courses of Rubidomycin from 2 to 3 mg/kg have caused a reduction of the leukaemic cell population in the bone marrow from 70 to 30% which has seemed sufficient to maintain her in acceptable health to enable her to continue her work as a housewife In spite of apparent failure of therapy some strikingly effec-

Table II Toxicity of Rubidomycin

Number of patients	20
Cardiotoxicity	10
Alopecia	4
Stomatitis	2
Peripheral neuropathy	1

tive actions on leukaemic skin lesions have been obtained In case 18, 2 large necrotic ulcerated lesions on the arm, 12 and 8 cm in diameter, healed completely within 14 days although there was no evidence of haematological remission

Toxicity

The toxic effects of Rubidomycin, excluding those on to the marrow, are shown in table II

Cardiotoxicity Patients on therapy with Rubidomycin were kept at bed rest for 48 h following injections of Rubidomycin and were examined regularly for signs of heart failure Routine ECG were carried out twice weekly No death from acute cardiac failure attributable to the drug was encountered in this series One patient (case 16) aged 71 years had a series of attacks of left ventricular failure after having received 9 5 mg/kg in intermittent dosage over 10 days but recovered on standard therapy with digitalis and diuretics

The ECG of patients under treatment were examined for 3 main features, these being arrhythmias, variations in form of the complexes and reduction in voltage Multifocal ventricular ectopic beats, occurring more frequently than 1 in 10 beats, developed in 2 patients (cases 5 and 12) Depression of the ST segments with T wave inversion developed in cases 5, 13, 16 and 20 and generalised T wave inversion was seen in cases 12 and 15 These electrocardiographic alterations were often prominent after 2–5 mg/kg doses of Rubidomycin and reverted to normal after 48 h The reduction in voltage was detected in similar circumstances in cases 7, 12, 13, 14 and 20 The most striking feature in the pattern of cardiotoxicity *in this series has been the relationship to age* Of the 9 patients aged less than 50 years who received doses of Rubidomycin varying from 125 to 1,200 mg (mean 425) only 1 showed signs of cardiotoxicity In the 11 patients aged more than 50 years, 8 showed

electrocardiographic changes In this group the dosages were similar with a range of 275–660 mg (mean 439)

Alopecia 4 of the patients, all over the age of 60 years, developed severe alopecia but in those patients who have remained in complete remission there has been satisfactory regrowth of hair of normal colour.

Neuropathy Routine and detailed examinations of the central nervous system were not carried out but 1 patient complained of paraesthesiae of hands and feet although no objective physical signs were detected This has continued even though he has received no further therapy and he has been in remission for several months

Discussion

Despite its considerable toxicity rubidomycin appears to be the most effective single drug for the induction of complete remission in adult myeloblastic leukaemia, a disease in which treatment previously has been disappointingly difficult [12] Complete remission rates of between 15 and 50% have now been obtained in adults by a number of workers [2, 4, 5, 10, 15] When used alone or in combination with steroids the rate of complete remission is greater than in other earlier induction treatments, many of which have involved the use of several drugs At this relatively early stage it is difficult to define the factors which auger well for remission but the vast majority of cases show sensitivity to the drug and true resistance seems rare BOIRON *et al* [2] have shown the drug to be more effective in the first attack than in subsequent relapses and have some evidence to indicate that children with acute myeloblastic leukaemia respond better than do adults

Widespread use of Rubidomycin in adult leukaemia has probably to be limited by what SCOTT [13] has termed as its brutal effect on the bone marrow In some of the earlier series appreciable numbers of patients died from fatal marrow aplasia and BERNARD *et al* [1] had 19 deaths due to this cause in 61 patients Although transient marrow aplasia seems to occur in the majority of patients treated it does not seem to be essential for the induction of complete remission and as with SOUHAMI and PRANKERD [15] we have found that smaller dosage regimes have reduced the deaths due to marrow aplasia It seems probable that, although the overall remission rate may be lower, this may be countered by the lower death rate from aplasia

The other serious effect of rubidomycin is that of cardiotoxicity Reports of this have varied greatly and there are obviously multiple factors concerned It has been suggested that cardiac accidents rarely occur when less than 750 mg/m² have been given [9] but MALPAS and SCOTT [10] showed cardiotoxicity after a total dose of 300 mg and congestive failure after a dose of 600 mg These latter workers felt that intermittent dosage regimes were less toxic BONADONNA and MONFARDINI [3] were also in favour of intermittent dosage regimes and in their small series of 16 adults death from acute cardiac failure occurred in 5 patients with dosages ranging from 2 2 to 8 0 mg/kg They considered cardiotoxicity to be dose dependent in children but not so in adults The low dose cardiotoxicity in middle aged and elderly patients has also been stressed by MARMONT *et al* [11] In the present series no deaths have been directly attributable to cardiotoxicity but the majority of patients have had intermittent drug therapy The ECG changes, recorded in detail in all patients, confirm the apparent toxicity in the elderly but do not show a direct correlation with cumulative dosage

Alopecia seems to be a constant feature in most series being present in 70% of BOIRON's cases [2] SOUHAMI and PRANKERD [15] found that they could eliminate this side effect by the use of occlusive elastic bands above the ears immediately before and half an hour after the injection of Rubidomycin but other workers have not recommended this Other various side effects have also been reported and include stomatitis, oral ulcers and vomiting [8], severe neurotoxicity [11] and cellulitis at the injection site [8] However, these seem relatively infrequent and do not pose basic problems in the management of the patients

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Acta haemat 46 74-91 (1971)

Clinical Experiences with Cytotoxic Immunosuppressive Treatment of Idiopathic Thrombocytopenic Purpura

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Key Words
Immunosuppression
ITP

Abstract After a review of the literature a series of 21 patients with ITP treated with non steroidal immunodepressive agents is presented The agents were azathioprine (AZP) methotrexate (MTX) cyclophosphamide (CPH) vinblastine (VLB) and L asparaginase (L ase) No permanent, unmaintained remission in chronic self perpetuating cases of ITP could be obtained even after splenectomy However, AZP was useful in the treatment of chronic cases either alone or in combination with steroids where it displayed a useful steroid sparing effect. CPH and MTX were generally ineffective VLB induced striking temporary thrombocytoses and L ase was followed by an early thrombocytosis but proved too toxic to be employed consistently Single case reports and remission percentages are reported

Although the demonstration of cell-bound and/or serum anti platelet antibodies in idiopathic thrombocytopenic purpura (ITP) is – differently from anti erythrocyte antibodies – fraught with difficulties, it is generally *recognized that most cases of the chronic type belong to an autoimmune* condition, as evidenced by the existence of (a) serum thrombocytopenic factors [22] which are 7 S [51] γG-immunoglobulins [23] and (b) by the blastic transformation of lymphocytes when reacted with autologous platelets [39] Indeed, when analyzed in terms of immunocyte (lymphocyte) population involved [43], ITP might well be classified as a disorder of the B lymphocytes

Although the majority of cases respond to corticosteroids and/or splenectomy, in the last decade a new approach to treatment has been made with the introduction of cytotoxic immunosuppressive agents Antipurine substances such as 6 mercaptopurine (6 MP), 6 thioguanine (6 TG) and azathioprine (AZP) were employed originally, but antifolinic, alkylating

and stathmokinetic agents such as methotrexate (MTX), cyclophospha mide (CPH) and vinblastine (VLB) have also been used more recently

Although remissions in single cases have been reported in the early literature no attempt to cover them will be made here because of space lim itations and the greater significance of larger clinical materials In 1964, DAMESHEK [15] first reported 2 subsequent remissions in each of 3 out of 14 cases shortly after the use of either 6-MP or one of its analogues In 8 other cases 'some beneficial effect' took place, while in 3 other cases there were none at all DAMESHEK [15] commented at that time that the inconstant effects of the antimetabolites had been 'somewhat discouraging' The Boston clinical material was subsequently enlarged by SWANSON and SCHWARTZ [55] and more extensively by BALDINI [3, 5], whose most recent observations include 16 cases, all treated with AZP, with a complete remission only in one case Antimetabolite therapy had been reserved for patients in whom conventional treatments (corticosteroids, splenectomy) had failed

In 1966, CORLEY *et al* [13] reported on AZP treatment of autoimmune diseases 14 cases of ITP were initially enrolled in this study but only 7 were considered eval uable Of these 3 attained normal platelet levels and one had a partial response Of the 3 complete remitters one died of pulmonary embolism while still on treatment, and the other 2 required maintenance treatment

In 1967, SUSSMAN [54] treated with AZP 8 patients with chronic 'refractory ITP Three of these patients had undergone splenectomy with no improvement of their condition and all had had long term corticosteroid treatment previously with occas onal severe side-effects (diabetes in 2 instances collapsed vertebra and psychic disturbance in one respectively) Haemostatic levels of blood platelets were obtained in 7 induction treatment was given at a dosage ranging from 3 to 6 mg/kg daily maintenance therapy with smaller doses was necessary

BOURONCLE and DOAN published their first report in 1966 [9] and subsequently enlarged their clinical material in 1969 [10] the latter figures will be referred to in this review Seventeen patients with refractory ITP, 7 males and 10 females 14 of whom had been already splenectomized and had relapsed, were treated with the equivalent of 1.2–2.4 mg/kg of AZP for periods ranging from 2 to 3 months. The response to therapy was graded as excellent, good fair and failure *excellent* indi cating a complete hematologic and clinical remission with no relapse after discon tinuation of all therapy *good* the same response but with the need of maintenance therapy *fair* some definite improvement with subnormal values and wide fluctua tion in spite of continued therapy *failure* no change in the platelet level and/or clin ical manifestations Five out of 17 patients treated with AP had an excellent response 7 had a good response 2 were considered to have made a fair response and 3 were failures.

Always with AZP 2 improvements out of 3 cases were reported by MIESCHER [36] and 3 out of 4 by LO *et al* [28] these last having been observed in children A

combination of AZP and prednisone was employed in a pediatric clinical material by KUZEMKI and KEIDAN [25]

Twelve patients with ITP were treated with 6-MP (11 cases) and AZP (1 case) by STIEGLITZ *et al* [53] in a report which was published in 1969, no differences between the clinical effects of these two antimetabolites could be noticed A variable increase in the platelet count was obtained in 7 cases but all required maintenance treatment.

Out of 341 cases of ITP treated at the Hopital Saint Louis of Paris [16, 17] 19 received cytotoxic immunosuppressive therapy, in the majority of cases these were persistent cases, refractory to steroids and having relapsed after splenectomy The drugs utilized were 6-MP, AZP, chlorambucil and VLB A complete persistent remission after AZP could be obtained in one splenectomized case, while in 3 other cases remission was transient, necessitating maintenance or even relapsing notwithstanding the same In a subsequent report [16] a significant increase of platelets after one injection of VLB was observed in 6 out of 10 cases, the chronology and magnitude of the platelet peak was comparable to our personal results as shown in figure 5

Also in 1970, MASSIMO *et al* [34] reported definite improvements in 4 out of 10 pediatric cases treated with AP

Finally, 11 patients previously treated with corticosteroids 9 of which had been already splenectomized and relapsed, were treated with CPH by LAROS and PENNER [26] The drug was administered in daily doses of 1–2 mg/kg and the dosage was increased in 50-mg increments every 1–2 weeks to a maximum of 4 mg/kg/day, or as limited by marrow toxicity When remission occurred CPH was gradually tapered and finally discontinued, the duration of treatment having ranged from 1 2 to 8 months Seven of these patients had an *excellent* response, with unmaintained remissions lasting from 10 to 40 months and 4 had *fair* responses, according to BOURONCLE and DOAN s criteria [9, 10]

Clinical Material and Methods

A total of 21 patients (5 male and 16 female) were evaluated for the results of immunodepressive therapy The ages ranged from 11 to 76 but only 2 were under 20 The diagnosis of ITP was made on the basis of history, clinical findings, haematologic examinations and bone marrow studies Platelets were enumerated all through this study by the FEISSLY LÜDIN [18] phase contrast method as modified in our laboratory [32] A mean of 235 000 platelets/mm³ is found by this method in the capillary blood of the healthy adult, with a coefficient of variation (CV) = ±5 30%

Only patients with the idiopathic form of the disease were included in this study However, cases 8 and 17 had an associated rheumatoid like syndrome and case 10 had a concomitant strongly positive (++++) direct Coombs test (IgG K incomplete haemagglutinin) with a weekly positive (+) antinuclear test these cases were never found to have a positive LE phenomenon and were not considered to fit – at least at that time – with a definite diagnosis of systemic lupus (SLE) Eight cases had

Table I

Case No	Age (1970)	Sex	Duration of disease	Previous therapy	Main cytotoxic drug	Results	Subsequent (and concomitant) therapy	Current therapy	Present status[1]
1	62	F	26 y	corticosteroids	AZP	good	corticosteroids	corticosteroids, AZP	MIR
2	28	F	6 m	corticosteroids, splenectomy	AZP	fair	corticosteroids	corticosteroids, AZP	MIR
3	37	F	34 y	corticosteroids, splenectomy	AZP	good to fair	corticosteroids, MTX, CPH, VLB	corticosteroids, AZP	PF
4	27	F	3 m	none	AZP	fair	corticosteroids, splenectomy	none	UCR
5	28	F	5 m	corticosteroids	AZP	good	corticosteroids	corticosteroids, AZP	MCR
6	40	F	10 y	corticosteroids	AZP	fair	corticosteroids	AZP	MIR
7	69	F	3 y	corticosteroids, CPH	AZP	failure	corticosteroids	corticosteroids, AZP	PF
8	56	F	4 m	corticosteroids	AZP	fair	corticosteroids	corticosteroids, AZP	MIR
9	62	F	2 y	corticosteroids	AZP	good	corticosteroids	corticosteroids, AZP	MCR
10	11	M	5 m	corticosteroids	AZP	good	corticosteroids	no follow up	
11	60	M	8 m	corticosteroids	AZP	good	corticosteroids, AZP	VLB	MIR
12	16	M	3 m	corticosteroids	MTX	excellent	none	none	UCR
13	27	M	21 y	corticosteroids	MTX	failure	corticosteroids	corticosteroids	MCR
14	56	F	3 y	corticosteroids	MTX	failure	corticosteroids, splenectomy	corticosteroids, AZP	MIR
15	31	F	30 y	corticosteroids, L-ase	VLB	good	splenectomy	none	UCR
16	30	F	1 m	corticosteroids, L-ase	VLB	fair	splenectomy	none	UCR
17	56	F	4 m	corticosteroids	VLB	failure	corticosteroids, AZP	corticosteroids, AZP	Not evaluable
18	35	F	4 m	corticosteroids	CPH	failure	AZP, splenectomy	none	UCR
19	74	F	3 m	corticosteroids	VLB	excellent	corticosteroids	none	UCR
20	76	F	3 m	corticosteroids	VLB	failure	corticosteroids	corticosteroids	MIR
21	71	M	15 y	corticosteroids	VLB	fair	corticosteroids	corticosteroids	MIR

[1] MIR Maintained incomplete remission, PF partial failure, UCR unmaintained complete remission, MCR maintained complete re-

Table II Results of 36 cytotoxic immunosuppressive courses in 21 patients with ITP

Agent	Courses	Results excellent	good	fair	failures
AZP	17	–	6 (2)[1]	5 (2)[1]	6 (1)[1]
MTX	4	1	–	1	2
CPH	3	–	–	–	3
VLB[2]	9	1	3	2	3
L-ase	3	–	–	3	–

[1] In splenectomized cases
[2] Each course composed of 3 to 9 injections

been suffering with purpura for more than 2 years before starting immunosuppressive treatment (and 4 out of these for more than 20 years) 7 for between 3 months and 2 years and 6 for between 1 and 3 months

Before immunosuppressive treatment was initiated, all but 2 patients had received corticosteroids, and 2 had been splenectomized Other 3 cases were subsequently splenectomized and thereafter received an immunosuppressive treatment different from the one listed under the heading 'main cytotoxic drug' on table I the result of these subsequent treatments are evaluated under the heading 'present status'

The immunosuppressive agents employed in this study were AZP, in doses comprised between 1 and 4 mg/kg daily, MTX (30 mg/m² twice or once weekly) CPH (200 mg daily up to totals of 8–10 g), VLB (0 10–0 15/kg weekly) and L ase (an E C 2 preparation kindly supplied by Farbenfabriken Bayer AG) at the dosage of 15,000 IU/m² Following BOURONCLE and DOAN s [10] criteria, responses to therapy were graded as excellent, good fair and failures, as specified below

Excellent complete stable remission even after discontinuation of treatment Good complete remission but necessitating maintenance treatment Fair some definite improvement of platelet levels but no normalization and wide fluctuation in spite of continued therapy *Failure no change in platelet levels*

Finally, the present status of patients was evaluated as maintained (M) or unmaintained (U), complete (C) or incomplete (I) remission (R) and/or persistent failures (PF) A breakdown of the overall clinical material is given on table I while table II lists the single courses performed and the results obtained with each agent

Results

The majority of patients were treated with AZP Out of 17 cases, good results were obtained in 6, 2 of which were previously splenectomized, fair results in 5 others, 2 of which were splenectomized, while another 6

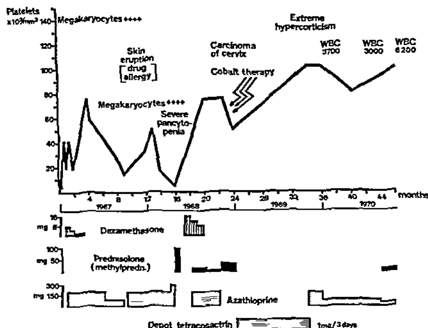


Fig 1 Case 1 combined corticosteroid-cytotoxic treatment of an inoperable case of chronic ITP

cases were attended by total failures The pattern of platelet increment in the responsive cases was gradual and generally less abrupt than the one following the administration of corticosteroids Toxic effects consisted in leukopenia which in some cases obliged to reduce dosage, reversal of leukopenia was then prompt, and no permanent damage to granulocytopoiesis was noticed In addition 2 elderly patients who had already been treated with corticosteroids for a long time developed prohibitive gastric intolerance No patient ever attained an unmaintained remission Two short reports of demonstrative cases (in either sense) follow

Case 1 (fig 1) was an obese (95 kg) hypertensive (200/120 mm Hg) moderately hyperglycemic (125 mg%) 62 year-old woman who had been suffering with chronic purpura and persistent thrombocytopenia for 26 years. She had not been previously advised to have her spleen removed and when first seen by one of us (A.M) in 1967 she was considered as a poor risk for splenectomy She first had a good response to dexamethasone, but developed hyperglycemia and glycosuria. She was accordingly put on AZP in the summer of 1967 she had a good response but

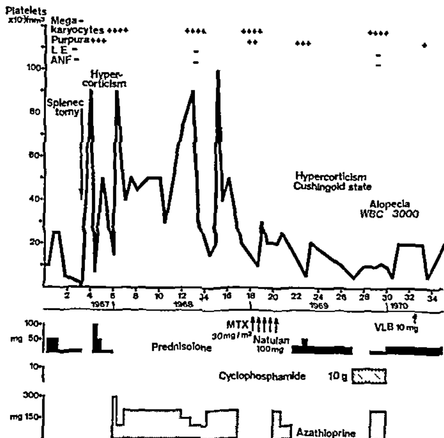


Fig 2 Case 3, refractory ITP relapsing after splenectomy and growing gradually resistant to all treatments

promptly relapsed on discontinuation of the drug because of a supposed drug allergy She had a second good response after readministration of the drug, which had to be discontinued again because of pancytopenia She did well again on AZP for many months, but then developed carcinoma of the cervix and was switched to steroids and tolbutamide during telecobaltotherapy She subsequently went into severe hypercorticism with depository tetracosactrin, and then received AZP all along 1970, but developed again leukopenia, which reached a low of 3,000 She was then put on a combination of *50* mg *AP* and *8* mg methylprednisolone/day, and after *4* months of this regimen was found to have 100,000 platelets, 6,200 leukocytes and 41% hematocrit, 105 mg% fasting blood sugar and no purpura

Case 3 (fig 2) concerned a 37 year old nulliparous woman with severe purpura and thrombocytopenia lasting practically from infancy, who had been splenectomized at the end of 1967, relapsed shortly thereafter, reacted favorably to steroids but went into hypercorticism, and then had a nice sustained remission of almost 1 year with AZP, but then relapsed while on a maintenance dose of 200 mg/day Her bone

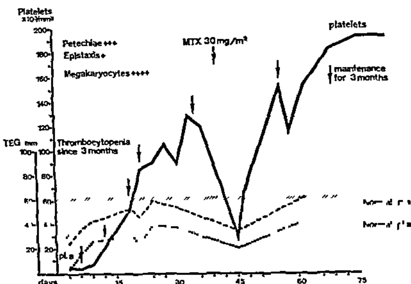


Fig 3 Case 12, ITP responding favorably to intravenous MTX. The shaded areas indicate the normal range for maximal amplitude and platelet activity as de termined thrombodynamically

marrow was repeatedly examined at that time, but no diminution of megakaryocytes suggestive of a mega suppressive effect of AZP could be detected The patient was subsequently treated with MTX, procarbazide CPH and VLB but the responses were extremely poor when not entirely negative This patient, who is on a perma nent steroid maintenance, because she otherwise develops recurrent severe metror rhages, has been evaluated as persistent failure (PF) A study with DFP[32] tagged platelets (performed in the Isotope Laboratory of the University Medical Clinic) showed a markedly shortened platelet survival

Out of 4 patients treated with parenteral MTX, one experienced a complete stable remission, while the others were practically unresponsive A short description of the responsive case follows

Case 12 (fig 3) concerned a 16-year-old male with an ITP who responded very gratifyingly to MTX administered at first weekly and subsequently at spaced inter vals. It could be noticed that there gradually appeared an early thrombocytopenia fol *lowing each injection, then attended by a rise The early amelioration of the platelet* clot retracting activity as determined thrombodynamically by the evaluation of the so-called 'platelet amplitude is also shown on the chart. This patient went on to unmaintained complete remission (UCR) and has never relapsed s nce

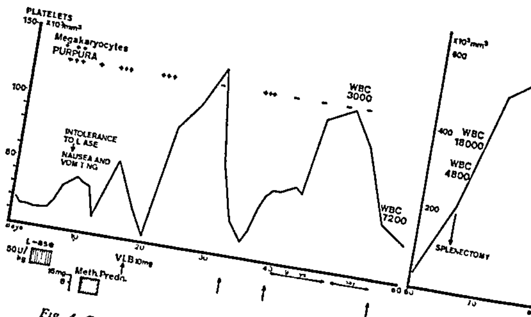


Fig 4 Case 15, ITP responding firstly to L ase, then to corticosteroids and subsequently to VLB Complete unmaintained remission after splenectomy

Three cases were treated with CPH in total dosages well over 8 g but none responded One of these (case 3) was undoubtedly the most refrac tory of our clinical material The second (case 5) achieved complete un maintained remission after splenectomy In the third case (case 18), se vere granulocytopenia with leukopenia (WBC 400/mm³) took place after 3 g administered by mouth over a period of 20 days, following 6 8 g of AZP over a period of 23 days This granulocytopenia reversed promptly after discontinuation of the drug She then went into unmaintained complete remission (UCR) after splenectomy

Nine cases were treated with VLB, of the 3 non responders the first was the already mentioned 'persistent failure' (case 3), while the second (case 17) was associated with rheumatoid arthritis of many years' duration, and is apparently refractory also to corticosteroids A brief outline of 2 responding cases follows

Case 15 was a 31 year-old nulliparous woman who remembered having suffered from cutaneous purpura, epistaxis and minor bleedings from infancy Ten years ago she had massive metrorrhagia, and was recognized to suffer with a severe thrombocytopenia. In addition she had had irregular and deficient menses since the age

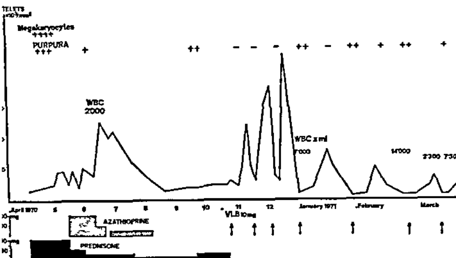


Fig 5 Case 11, ITP responding firstly to AZP and subsequently to VLB The characteristic see-saw pattern of platelet response to VLB in ITP is well apparent.

of 14, and was completely amenorrheic for the last 5 years She was moderately hirsute, and celioscopy during this hospitalization showed polycystic ovaries consistent with Stein Leventhal s syndrome After a diagnosis of ITP could be established in June 1970 on the basis of blood and bone marrow examinations, she was treated as an outpatient with 8 mg of methylprednisolone daily for 3 months, with an increase of her blood platelets from 5 000 to 20 000–25 000 throughout this period. The cutaneous purpura subsided significantly She was hospitalized on September 17, 1970 steroids were withdrawn and a slight decrease of platelets started to take place (fig 4 beginning of chart) She was given 15 000 IU/m² (500 IU/kg) of L-ase for 3 days, after which treatment had to be discontinued because of severe nausea and vomiting, however the platelets had already reached 38 000 She was then given 8 mg of methylprednisolone, and had an additional increase to 58 000 Steroids were discontinued and after 3 days she was given 10 mg of VLB intravenously, platelets were 21 000 at that time They dropped brusquely to 4 000 after 48 h and epistaxis with cutaneous purpura developed. However, these clinical manifestations subsided rapidly and a peak up to 140 000 platelets took place, the peak having been reached 9 days after VLB injection There followed a rather precipitous fall of platelets at the level of 27 000, another 10 mg of VLB were given, followed firstly by an ulterior fall to 16 000 on the third day, and subsequently by a second peak of 125 000 9 days after the injection A leukopenia of 3 000 WBC/mm³ prevented ulterior administration of VLB at this time. There followed a subsequent fall to 43 000, when another 10 mg of VLB were given, with a subsequent rise to 112,000 It was decided to have the spleen removed during this 'remission wave splenecto-

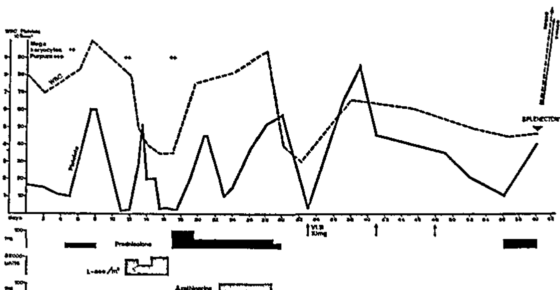


Fig 6 Case 15, ITP responding subsequently to steroids, L ase, steroids and AZP, VLB and finally splenectomized Platelet increment after splenectomy was particularly prompt

my was performed on November 11, 1970, at a time when the blood platelets were 110,000 and was followed by a rise to 215,000 after 24 h, and to a peak of 625,000 15 days after operation The patient is in unmaintained complete remission (UCR) at present

Case 11 (fig 5) is a 60-year old catholic priest, who started to suffer with gingival oozing and petechiae in April, 1970 At that time his blood platelets were 15,000, and his bone marrow was thought compatible with ITP He was treated initially with 100 mg/day of prednisone, but his platelets, after attaining 50,000 after 5 days, went back to 30,000 AZP (200 mg/day for 25 days, and thereafter 100 mg/day for another 7 days) was added and prednisone was gradually tapered off, platelets attained a peak of 126,000, but WBC went down to 2,000 The patient was subsequently carried on with minor adjustments both of AZP and prednisone, until he was put on VLB alone, starting on November 13, 1970 Each of the administrations was followed by a sharp rise and fall of the platelets, following the pattern shown on the chart Each thrombocytosis was synchronous with leukopenia The latest administrations have been, however, attended by a decreasing augmentation of thrombocytes and an increased lowering of leukocytes, suggesting a progressive loss of effect on the thrombocytopenia and an increasing myelosuppression

The case history of a third VLB-responder is reported with the L-ase-treated group described below

All 3 patients treated with L-ase (cases 5, 15 and 16) had a moderate but extremely prompt increase of circulating platelets, respectively from

12,000 to 55,000, from 15,000 to 31,000 and from 2,000 to 55,000 In all 3 cases the treatment had to be discontinued because of severe diges tive toxicity (gastric and hepatic), while in cases 5 and 15 the fall of platelet levels took place immediately after discontinuation, in case 16 this happened still during treatment (fig 6) A brief description of this last case follows

Case 16 (fig 6) was a 30-year-old woman who started suffering with thrombocytopenic purpura about one month before hospitalization. Her blood and bone marrow findings were consistent with a diagnosis of ITP She was given 25 mg/day of prednisolone, and had a first response with a high of 60 000 platelets, after a start from 10 000 Discontinuation of treatment was followed by a withdrawal re lapse to 2 000, after which she received L ase for 6 days, when treatment had to be discontinued because of severe gastrointestinal and hepatic toxicity The platelets made a steep rise to 55,000, but started to fall at the first dosage reduction, and failed to increase even after the augmentation of dosage She subsequently had an other good response to corticosteroids grew increasingly Cushingoid had a new par tial relapse after reduction of dosage and responded again to the addition of AZP She was subsequently allowed to relapse and went on to a nadir of 4 000 She then received 10 mg of VLB and a peak of 85 000 after 8 days but thereafter failed to respond to another two administrations of the alkaloid Her spleen was removed on December 18, 1970 Her platelets rose from 36 000 to 216 000 after 6 h from sple nectomy and to 596 000 after 6 days. She is now in unmaintained complete remis sion (UCR)

Discussion

Summing up our clinical material it may be readily seen that an 'excellent' result that [illegible]

that, also in view of the absence of relapses, we feel it should not be con sidered as a chronic form of ITP In all the truly chronic cases relapses invariably followed the discontinuation of treatment, as already remarked by others Apparently, permanent unmaintained remissions in splenectomized, relapsing, chronic cases of ITP were observed in only one series [9, 10] However, our observations, consistent with those of others [3, 5, 15, 16], do not reflect disfavourably on the cytotoxic immunosuppressive agents, since it is known that thrombocytopenia will generally return also after corticosteroids in the chronic, self perpetuating forms of the disease, when the medication is withdrawn [3, 14, 31] Still, even with such limita-

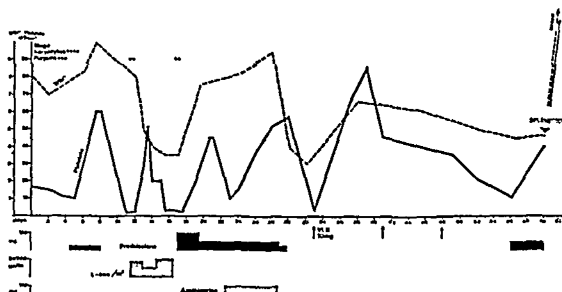


Fig. 6. Case 15, ITP responding subsequently to steroids, L-ase steroids and AZP, VLB and finally splenectomized. Platelet increment after splenectomy was particularly prompt.

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Discussion

Summing up our clinical material it may be readily seen that an 'excellent' result – that is unmaintained complete remission (UCR), practically equivalent to a 'cure' – was obtained in only 2 patients (cases 12 and 19) In these cases the disease had been lasting for not more than 3 months, so that, also in view of the absence of relapses, we feel it should not be considered as a chronic form of ITP In all the truly chronic cases relapses invariably followed the discontinuation of treatment, as already remarked by others Apparently, permanent unmaintained remissions in splenectomized, relapsing, chronic cases of ITP were observed in only one series [9, 10] However, our observations, consistent with those of others [3, 5, 15, 16], do not reflect disfavourably on the cytotoxic immunosuppressive agents, since it is known that thrombocytopenia will generally return also after corticosteroids in the chronic, self perpetuating forms of the disease, when the medication is withdrawn [3, 14, 31] Still, even with such limita-

tions, AZP has proved its usefulness also in our hands in cases complicated with diabetes, corticosteroid side-effects and the other situations outlined by SUSSMAN [54]

A methopterin (MTX) has perhaps not been fully exploited in refractory ITP Although we have obtained a clear result only in an obviously self-limited case, it should be also remarked that the dosage adopted in the other, self perpetuating cases was certainly too low In view of its specific activity on the division of immunoblasts rather than on blastogenesis [56], its use in higher doses, perhaps also in combination with folinic 'rescue' [7], might be warranted

CPH has recently received an enthusiastic evaluation [26], having produced long lasting (up to 40 months), unmaintained remissions Although our failures (3 cases) are too few to challenge this evaluation, they do, however, seem to request additional clinical evidence

Although a thrombocytosis-producing effect had been already noticed in sporadic cases of chronic lymphatic leukemia and Hodgkin's disease treated with Vinca alkaloids [41], VLB was employed for the first time in the treatment of chronic ITP by DELOBEL *et al* [16, 17], who obtained a significant rise of platelets after a single injection in 6 out of 10 cases, in their studies, they found that at maximum peak platelet aggregation, ADP release and platelet factor 3 formation were normal, but that platelet life span was still shortened A direct activity on the megakaryocytes with increased production was accordingly postulated[1]

In those cases of ours in which a response was obtained, its pattern was closely similar to the one reported by the French investigators [16 17], the peak of platelet increment generally occurring on the ninth day after the administration, in addition, a moderate fall in the first few days after administration was often observed, and was interpreted as the effect of an early myelosuppression upon the megakaryocyte lineage

Not having performed platelet survival determinations before and after the alkaloid's administration, we cannot offer a definite theory on the mechanism of action of VLB in ITP It is known that the germane alkaloid vincristine has been shown to induce thrombocytosis in intraperitoneally injected mice [42], and to selectively destroy hyaloplasmic microtubules [57] and the circumferential band of platelets [58], thus impairing their secondary aggregation [59] and perhaps lengthening their survival

[1] A normal platelet lifespan in VLB remitters has been however reported subsequently by the same group of investigators [SULTAN Y, DELOBEL, J JEANNEAU C. and CAEN J P *Lancet I* 496–497 (1971)]

[52] However, such a mechanism, though conceivably operative for the VCR induced thrombocytosis in normal mice, would not be expected to exercise significant effects in human patients with markedly enhanced random platelet destruction and already maximally increased platelet production [20, 21] Here, the hypothesis that VLB, which is already known to be a potent immunosuppressive agent [1], by interfering with the immunocyte mitotic cycle and/or other mechanisms which have been reviewed elsewhere [33], is acting by inhibiting platelet antibody biosynthesis, still appears to be the most probable In any case, VLB-induced thrombocytosis in ITP appears significant enough to warrant its introduction in clinical haematology in selected cases

The enzyme L ase has been investigated by us in some pilot experiments in ITP, in view of the recent demonstration of its marked immunosuppressive activity [12, 24, 29, 40, 45], a finding which is in agreement with the exogenous asparagine-dependency of immature lymphoid cells Thus, it could be shown [2, 11, 24, 37] that L ase strongly suppresses PHA- and antigen induced lymphocyte blastogenesis, homograft reactivity [8, 27, 46] and other phenomena of cell-bound immunity, but also elicits a severe depression of the production of humoral antibodies such as heterologous haemagglutinins and haemolytic plaque formation [12, 29, 40, 49] In addition, adjuvant-induced arthritis in the rat is inhibited [29], and some therapeutic effects on connective tissue diseases were also observed [19] In our hands, although it was clear that the transient and moderate type of response obtained, together with the severity of side-effects, does not justify the inclusion of L-ase in the practical therapeutic armamentarium of ITP, it would seem that an appreciable degree of immunodepression might have taken place, thus determining a certain augmentation of platelets That this may be a probable mechanism is also supported by the fact that, in the nonthrombocytopenic patients, a variable and sometimes marked thrombocytopenia generally will follow treatment with this enzyme [30], thus making the early thrombocytosis in ITP even more significant

In conclusion, AZP certainly remains the leading immunodepressive cytotoxic agent of ITP Its use is most advantageous in cases displaying severe side-effects to corticosteroids and in diabetic and/or obese patients Also for the postsplenectomy relapsing patients a steroid-AZP combination appears to hold most advantages, each drug enabling to 'spare' the other However, AZP is definitively not the answer for the really refractory cases, which would seem to make up a category of 'malignant' ITP

Our experience both with MTX and CPH is meager In agreement with DELOBEL [16, 17] we should like to put VLB in the second place in the nonsteroidal medical treatment of ITP Its thrombocytosis-inducing effect, though still poorly understood, may be striking and its tolerance is generally good, so that it may become indeed useful in selected cases

Finally, the asparagine-depleting enzyme L-ase has been shown to exert a certain thrombocytosis inducing effect, probably by means of its immunosuppressive activity, although its practical implications remain scarce, also in consideration of its prohibitive toxicity in a nonleukaemic disease such as ITP

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Acta haemat 46 92–105 (1971)

The Haematological Response to the Immunosuppressive Methylhydrazine Derivates Procarbazine and Ro 4-6824

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Abstract Blood response patterns, lethality and weight loss were compared in skin allografted mice treated with the immunosuppressive methylhydrazine derivatives procarbazine and Ro 4-6824 These drugs acted on neutrophils similar as the cytotoxic agents of the 'mustard type', causing a rapid fall with prompt and overcompensatory replacement In contrast, the recovery from the severe lymphopenia was slow Thus the methylhydrazine may affect circulating mature neutrophils, but precursors of lymphocytes The results provide further support for a particular effect of methylhydrazine derivatives on lymphoid tissue If both agents were compared, weight loss, lethality and leucopenia were more marked with procarbazine while Ro 4-6824 diminished the circulating erythrocytes and thrombocytes more severely Supplementary ALS added little to the haematological and toxic effects

Key Words
Immunosuppression
Methylhydrazine derivatives
Procarbazine
Skin allograft

The methylhydrazine derivatives were introduced originally as cytotoxic agents [1], but it became soon apparent that they impaired immune responses [2, 3] and even belonged to the most effective immunosuppressive drugs [4] Their potency was comparable to antilymphocyte serum (ALS) [5] The immunosuppressive activity of the methylhydrazine derivatives is consistent with the observation that in the clinic, lymphoproliferative disorders display a preferential susceptibility to these drugs [6, 7]

The experimental studies on the immunosuppression by methylhydrazine derivatives were carried out mainly with 2 representatives of this class, namely procarbazine (1-methyl-2-*p*-isopropylcarbamoylbenzylhydrazine hydrochloride, Natulan®) and Ro 4-6824 (1-methyl-2-*p*-allophanoylbenzylhydrazine hydrobromide) Procarbazine is used clinically for the

treatment of malignant lymphoma With regard to immunosuppression, experimental results suggested that Ro 4-6824 had a slightly more selective immunosuppressive effect, prolonging skin allografts in mice and rabbits as well as procarbazine but with less toxicity [8] Moreover, the ratio immunosuppression/erythrocytotoxicity was higher with Ro 4-6824 [9]

As haematopoietic depression may be one of the factors limiting the effective immunosuppressive dosage of these drugs, the haematological responses to procarbazine and to Ro 4-6824 were compared

Methods

Experiment A

The drugs were tested in a dosage schedule which corresponded to their use for the induction of tolerance to skin allografts in mice [10] Accordingly, the treatment schedule included two doses of ALS

Recipients were male C3H mice (20–28 g) They were grafted by a standard technique [11] with tail skin from BALB/c donors A chronic and an acute schedule of drug administration were used The drug dosage was chosen to cause equal graft prolongation with both agents The chronic protocol consisted of 12 doses of 120 mg/kg procarbazine or of 150 mg/kg Ro 4-6824, injected every other day before the grafting and starting at day –23 In the acute protocol, 360 mg/kg procarbazine or 450 mg/kg Ro 4-6824 were injected daily on the 4 days preceding the transplantation. Control mice were grafted at day 0 and received no immunosuppressive treatment

Both procarbazine and Ro 4-6824 were dissolved immediately before the administration in distilled water (which was warmed to about 45 °C for Ro 4-6824) and injected by the subcutaneous route On days +1 and +5 after the grafting (day 0), 0.5 ml of ALS per mouse were applied by the same route The ALS was prepared in rabbits by a standard technique [12] It was Seitz-filtered and heated for 30 min to 56 °C. No absorption with mouse erythrocytes was carried out. Blood counts were performed with groups of 5 mice by severing the tail of the mice and with standard haemocytometer techniques

Experiment B

In this experiment, female A mice were treated with Ro 4-6824 only This strain tolerated higher doses of Ro 4-6824 than the C3H recipients and was pretreated during 4 weeks on 5 days weekly with 300 mg/kg Ro 4-6824 subcutaneously One day before the transplantation, 40×10^6 donor (CBA) spleen cells were injected intravenously Before the treatment was terminated the dose was reduced to 150 mg/kg of Ro 4-6824, applied for 7 days following the transplantation with CBA skin This experiment permitted to determine the relative contribution of ALS to the haematological responses seen in experiment A. One out of the 5 mice became permanently tolerant while the others rejected their grafts at 35–40 days [8] The controls received subcutaneously saline and grafts survived for 8–10 days

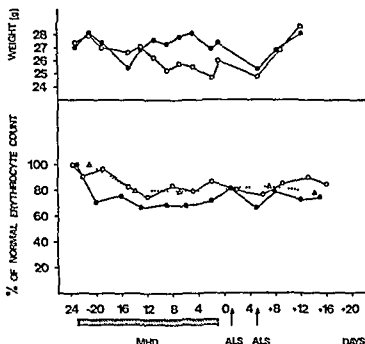


Fig 1 Erythrocyte response in C3H mice to a combined treatment of methyl hydrazine derivatives (MHD) and 2 injections of 0.5 ml ALS The mean values of 5 mice are shown *(a) Chronic protocol* 12 injections of either 120 mg/kg procarbazine of 150 mg/kg Ro 4-6824 on alternate days from day -23 to -1 ○ Procarbazine ● Ro 4-6824 △ controls

Results

Experiment A

Weight loss Figure 1a and b show that it was more pronounced with procarbazine and amounted to 10% with the chronic and to 20% with the acute protocol Ro 4 6824 caused chronically no weight loss and acutely one of less than 10% The weight loss – aggravated at day 0 by the operative procedure – was recovered promptly after cessation of the treatment

Lethality With the chronic protocol 10 out of 44 (22 8%) grafted mice died during the experiment if treated with 120 mg/kg procarbazine and 4 out of 25 (16%) treated with 150 mg/kg Ro 4-6824 (table I) As the additional treatment with 0 5 ml ALS on days +1 and +5 did not affect the lethality, mice treated with the methylhydrazine derivatives alone or together with ALS are pooled With the lower chronic regimen of 120 mg/kg Ro 4 6824 only one out of 44 mice died At this dosage

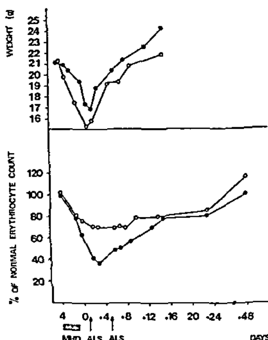


Fig 1b Acute protocol 4 daily injections of 360 mg/kg procarbazine or 450 mg/kg Ro 4-6824 on days –4 to –1 The weight (in g) is also presented. The mice were grafted with BALB c skin on day 0 The controls received subcutaneous injections of saline o Procarbazine • Ro 4-6824

Ro 4-6824 alone prolonged grafts to 14.5 days versus 16 4 days with 150 mg/kg Combined with ALS, 120 mg/kg of Ro 4-6824 were as effective as 150 mg/kg The administration of both drugs on 4 days (acute protocol) was tolerated without fatalities for 360 mg/kg/day of procarbazine (40 mice) and for 450 mg/kg/day of Ro 4-6824 (16 mice)

Erythrocytes and reticulocytes The response is shown in figure 1 and 2 The erythrocytes were moderately affected with the chronical protocol and slightly more by Ro 4-6824 than by procarbazine This was reflected by a reticulocytosis attaining fourfold increased values after 10 days of treatment with both drugs The acute protocol affected the erythrocytes more severely Ro 4-6824 caused a drop of more than 60% The reticulocytes rose 47 times with Ro 4 6824 and 23 times after procarbazine at about 11 days after the cessation of the treatment

Table I Lethality of C3H mice treated according to the chronic or acute protocol with equieffective doses of procarbazine or Ro 4-6824 Additional administration of twice 0 5 ml ALS did not increase the lethality

Treatment	ALS	MST[1], days	Lethality, number of mice/total number of mice
Controls	no	11.2	2/38
Controls	yes	29 3	0/26
Procarbazine, 120 mg/kg × 12	no	16 6	5/15
Procarbazine, 120 mg/kg × 12	yes	46.2	5/29
Ro 4-6824, 150 mg/kg × 12	no	16 4	2/10
Ro 4-6824, 150 mg/kg × 12	yes	38 3	2/15
Ro 4-6824, 120 mg/kg × 12	no	14 5	0/15
Ro 4-6824, 120 mg/kg × 12	yes	38 6	1/27
Procarbazine, 360 mg/kg × 4	no	17 3	0/15
Procarbazine, 360 mg/kg × 4	yes	56 3	0/25
Ro 4-6824, 450 mg/kg × 4	no	15 7	0/10
Ro 4-6824, 450 mg/kg × 4	yes	40 5	0/6
Ro 4 6824, 360 mg/kg × 4	no	13 9	0/15
Ro 4-6824, 360 mg/kg × 4	yes	42 5	0/15

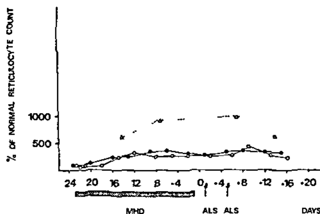
[1] MST, median survival time of BALB/c skin grafts

Thrombocytes With the chronic protocol, the group treated with Ro 4-6824 showed a moderate drop of platelets With procarbazine, a more pronounced fall of 60% was seen (fig 3a) After cessation of the treatment, an explosive overcompensatory recovery took place In the acute protocol, the thrombopenia was severe (fig 3b), with Ro 4 6824 a drop to 5% of the pretreatment value occurred However, no bleeding tendency was noticed and the recovery was rapid

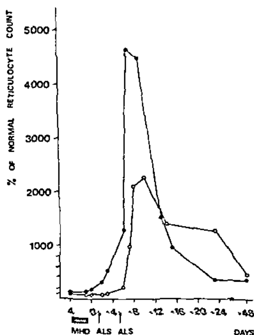
Leucocytes After the chronic treatment, the lymphopenia was slightly more marked with procarbazine while Ro 4-6824 led to a more consistent granulocytopenia (fig 4a) A compensatory neutrophilia was observed one week after the treatment was stopped In the acute protocol, both drugs caused very similar changes, i e a protracted lymphopenia of 3 weeks duration and a neutrophilia of remarkable intensity (4–5 times normal), (fig 4b)

Experiment B

Erythrocytes and reticulocytes The main drop of the erythrocytes oc-



a



b

Fig 2 Reticulocyte response Mice treated as in figure 1 Blood was taken from the tail vein of the controls twice weekly as from the experimental mice ○ Procarbazine ● Ro 4-6824 controls

Table I Lethality of C3H mice treated according to the chronic or acute protocol with equieffective doses of procarbazine or Ro 4-6824 Additional administration of twice 0 5 ml ALS did not increase the lethality

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Controls	yes	29 3	0/26
Procarbazine, 120 mg/kg × 12	no	16 6	5/15
Procarbazine, 120 mg/kg × 12	yes	46 2	5/29
Ro 4-6824, 150 mg/kg × 12	no	16 4	2/10
Ro 4-6824, 150 mg/kg × 12	yes	38 3	2/15
Ro 4-6824, 120 mg/kg × 12	no	14 5	0/15
Ro 4-6824, 120 mg/kg × 12	yes	38 6	1/27
Procarbazine, 360 mg/kg × 4	no	17 3	0/15
Procarbazine, 360 mg/kg × 4	yes	56 3	0/25
Ro 4-6824, 450 mg/kg × 4	no	15 7	0/10
Ro 4-6824, 450 mg/kg × 4	yes	40.5	0 6
Ro 4-6824, 360 mg/kg × 4	no	13 9	0/15
Ro 4-6824, 360 mg/kg × 4	yes	42.5	0/15

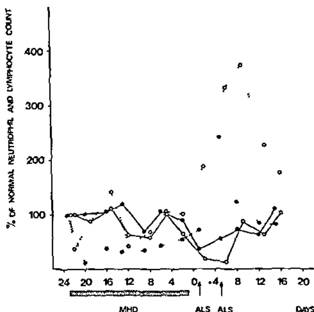
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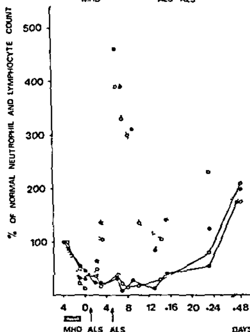
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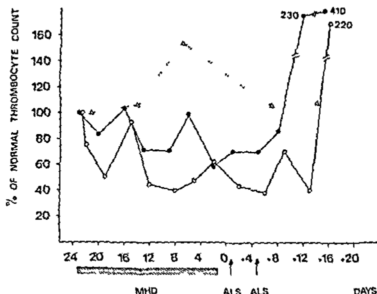


a



b

Fig 4 Response pattern of lymphocytes (solid lines) and neutrophils (broken lines)
See legend of figure 1 Procarbazine ● Ro 4-6924



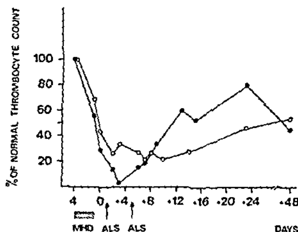


Fig 3 Thrombocyte response pattern See legend of figure 1 ○ Procarbazine, ● Ro 4 6824, △ controls

curred within the first 3 weeks of treatment (fig 5) It was accompanied by a gradual and simultaneous reticulocytosis In contrast to experiment A (fig 2a), the controls which were bled at about 2-week intervals only displayed no reticulocytosis

ambucil' – or 'nitrogen mustard-type [13], including cyclophosphamide [14] A less striking neutrophilia is also seen after single doses of sublethal X-irradiation Thus methylhydrazine derivatives may share some of the properties of alkylating agents or radiation However, unlike the neutrophils, the depression of the lymphocytes is prolonged The lymphopenia is both in line with the sensitivity of lymphoproliferative disease to these drugs and with the cellular depletion of lymph nodes and thymus seen after the treatment with Ro 4 6824 in mice [15] Although the methylhydrazine derivates do not only affect lymphocytes, the intensity and duration of their depression argue in favour of a relative specificity It would be interesting to study whether an individual population of lymphocytes (B- or T-cells) is specially sensitive

The inspection of the blood response pattern allows to compare the relative effects of the two methylhydrazine derivatives The examination of the drug-induced haematological response *sensu strictu* is complicated in our experiments by such additional measures as ALS, skin grafting, intravenous cell injection (in experiment B) and rejection The latter did not set in with both experiments (A and B) earlier than on day 35 and might have, if at all, influenced only the final stages of the counts

As to the ALS, the supplementary administration of twice 0 5 ml had hardly any effect on the haematological response pattern due to the *methylhydrazine derivatives* If the chronic pretreatment protocols of experiment A (with ALS) and experiment B (without ALS) are compared, it can be seen that ALS did not alter significantlv the response pattern, although it provided additional immunosuppression

The skin grafting procedure alone also did not cause impressive blood changes In particular separate control experiments (not presented) showed that untreated mice displayed after the grafting no neutrophilia as the animals in figures 4 and 7

However, it must be considered that the bleeding only of the mice from the tail tip at approximately 2 or 3-day intervals affects the blood count Thus some of the observed changes including perhaps the initial thrombopenia in figure 3b and the ensuing thrombocytosis (fig 6) may be related or partially due to the frequent removal of relatively substantial quantities of blood As a consequence, an element of caution should be introduced with regard to the interpretation of some haematological effects This does not apply however, to the *comparison* of the two drugs

Nevertheless the blood response pattern provides some indication as to the mode of action of the methylhydrazine derivatives With the

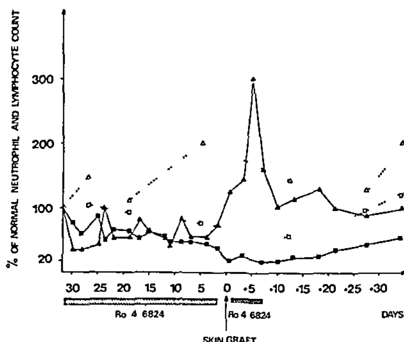


Fig 7 Response patterns of lymphocytes (■) and neutrophils (▲) Treatment as in figure 5 △ Neutrophils and □ lymphocytes of the controls

The pattern of the reticulocyte count may be affected by two opposite influences, namely the haemolytic stimulus and a depression of erythrocyte precursors With the dosage used in experiment A (fig 2a) the latter may prevail, whereas in experiment B a higher rate of haemolysis (and the less frequent bleeding of the [fig 5] controls) might account for the increase of the reticulocytes as compared to the controls

In the acute regimen, the thrombopenia was more severe with Ro 4-6824 The decline with the chronic protocol was followed by a striking compensatory thrombocytosis setting in after the treatment had been stopped or during the treatment (experiment B) This prompt replacement may explain why no bleeding occurred and is indicative of functional stem cells Methylhydrazine derivatives may, therefore, act on circulating platelets

A finding of some interest is the rapid recovery of the neutrophils after the cessation of the treatment The ensuing overcompensation is strongly reminiscent of the neutrophilia following the initial fall of granulocytes after a single dose of an alkylating cytotoxic agent of the 'chlor-

ambucil' – or 'nitrogen mustard' type [13], including cyclophosphamide [14] A less striking neutrophilia is also seen after single doses of sublethal X irradiation Thus methylhydrazine derivatives may share some of the properties of alkylating agents or radiation However, unlike the neutrophils, the depression of the lymphocytes is prolonged The lymphopenia is both in line with the sensitivity of lymphoproliferative disease to these drugs and with the cellular depletion of lymph nodes and thymus seen after the treatment with Ro 4-6824 in mice [15] Although the methylhydrazine derivates do not only affect lymphocytes, the intensity and duration of their depression argue in favour of a relative specificity It would be interesting to study whether an individual population of lymphocytes (B- or T-cells) is specially sensitive

The inspection of the blood response pattern allows to compare the relative effects of the two methylhydrazine derivatives The examination of the drug induced haematological response *sensu strictu* is complicated in our experiments by such additional measures as ALS, skin grafting intravenous cell injection (in experiment B) and rejection The latter did not set in with both experiments (A and B) earlier than on day 35 and might have, if at all influenced only the final stages of the counts

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Nevertheless the blood response pattern provides some indication as to the mode of action of the methylhydrazine derivatives With the

chronic protocol, the rapid recovery of thrombocytes and neutrophils points toward a more pronounced effect on these circulating mature elements than on the precursor cells. Particularly, the exaggerated 'mustard type' neutrophilia is indicative of functional stem cells [16]. Only the lymphocyte response displays a slow onset and protracted recovery which may indicate an effect on developing or precursor cells. This would also agree with biochemical observations according to which procarbazine inhibits the incorporation of nucleosides into (leukaemic) lymphocytes [17]. Further, the effect on precursor cells would explain why methylhydrazine derivatives are more effective if they are applied before the antigenic stimulus [2–5, 8, 18] and why they retard the recovery from ALS [19], which affects itself mature circulating lymphocytes. Their replacement from precursor elements would be prevented by the methylhydrazine derivatives.

If procarbazine and Ro 4 6824 are compared, the greater weight loss, lethality and leucopenia after the administration of procarbazine is balanced to some extent by the more severe erythrocytopenia and thrombocytopenia seen after Ro 4 6824. Hence, according to this study, both drugs seem to be of about equal suitability to be used as immunosuppressants on their own or more convincingly, to enhance the effects of ALS [10, 19].

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Acta haemat 46 106–120 (1971)

Inheritance of Haemoglobin H Disease

A new Aspect[1]

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Abstract A study of 23 patients with Hb H disease and their 82 relatives in 17 families showed that 2 types of this condition exist One is associated with the presence of a small slow moving component which we tentatively called the X component and which was invariably present in one parent Some siblings also had it The other type was not associated with this component Two patients without X component had a newborn with Bart's haemoglobin without X component None of the parents of 20 newborns with Hb Bart's without the X component had the X component It was present in only one parent of each of 2 newborns with Hb Bart's and the X component They are thought to represent Hb H disease in the newborn period We suggest that at least 3 abnormal genes may lead to Hb H disease, which results when 2 of the 3 combine Severity of clinical and haematological symptoms depends upon which abnormal gene is present and which 2 are involved in any particular combination

Key Words
α Thalassaemia
Haemoglobin Bart's
Haemoglobin H disease
Haemoglobinopathies

Despite much work on haemoglobin H disease and great efforts to unravel its mode of inheritance, the genetic basis of this condition is still obscure Many theories have been put forward, but proof for their correctness is still lacking Since Hb H consists solely of β-chains, it is thought that somehow the production of α-chains is depressed in this disease, leading to a surplus of β chains and the formation of Hb H (β_4) Indeed, CLEGG and WEATHERALL [2] reported evidence of this depressed production of α chains It is, therefore, thought that Hb H disease should be grouped with the category of α-thalassaemia, in which the production

[1] This work was supported by Research Grant 10486 by the University of California International Center for Medical Research and Training (UC ICMRT) with Research Grant AI-10051 both from the National Institutes of Health, US Public Health Service and by Research Grant WHO G3/181/1

of α-chains is depressed One theory currently believed to be the most acceptable is that Hb H disease results from inheriting 2 types of α thalassaemia traits, one severe and one mild, and that these traits are detectable during the neonatal period when babies with α-thalassaemia carry Hb Bart's [4, 5, 20]

In an attempt to throw more light on this intriguing problem, we examined carefully many patients with Hb H disease and their relatives, paying special attention to their haemoglobin patterns, and in this paper we present evidence that different types of genes are involved in producing the condition currently known as Hb H disease

Material and Methods

23 patients with Hb H disease and 82 relatives in 17 families were examined some had earlier been diagnosed as having the disease and had responded to our request to return for reexamination All had chronic haemolytic anaemia with typical intracellular Hb H inclusion bodies and their haemolysates contained Hb H Blood was obtained by venipuncture and placed in ethylenediaminetetraacetic acid (EDTA) for haematological study and in acid citrate dextrose (ACD) solution for haemoglobin analysis Serum was obtained from clotted blood for serum iron estimation A survey on cord blood was also carried out to detect newborns carrying Hb Bart s and the parents of babies with this component were examined

Haematological examinations followed standard methods Haemoglobin electrophoresis was performed on starch gel using tris-EDTA boric acid buffer pH 8 0 and pH 8 6 in discontinuous tris citrate boric acid buffer pH 9 5 and on cellulose acetate paper with tris-EDTA boric acid buffer pH 8 9 Haemoglobin components were stained with benzidine and o-dianisidine

Components were quantitated or isolated by diethylaminoethyl (DEAE) column chromatography using tris HCl buffers for the elution of the haemoglobin components [6] and by the cellulose acetate electrophoretic method [15] Alkali denaturation followed the method of Singer *et al* [17]

Results

Because clinical and haematological studies gave a wide range of findings that defied precise classification, we paid more attention to haemoglobin patterns using different buffers at different pH levels These proved more rewarding According to these patterns the patients could be divided into 2 groups In the biggest group the patients had in addition to haemoglobins A H and Bart's, a small haemoglobin component that we shall tentatively call X, which migrates more cathodically than Hb A_2 at alkaline pH In this group where both parents could be exam-

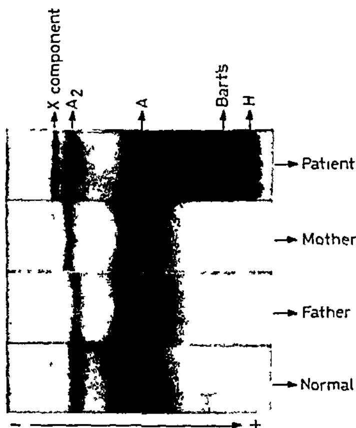


Fig 1 Cellulose acetate electrophoresis in tris EDTA boric acid buffer pH 8 9 showing the Hb pattern of a typical Hb H disease patient with an X component his mother s with the same component and his father s without that component compared with a normal control O dianisidine stain

ined, one parent of each subject studied invariably carried the same small slow-moving component in the blood, while the other did not (fig 1) One case (family No 7) at first seemed to be exceptional because, although the patient had the abnormal X component, neither parent did However, blood group studies revealed that the man believed to be the father of the patient could not have been The propositus had blood group A, the mother blood group O, and the father blood group B After close inquiry the mother admitted that the child was adopted after birth but she did not want this to be known In families where other members were available for study in addition to the parent having the abnormal Hb component several siblings also carried this abnormal-

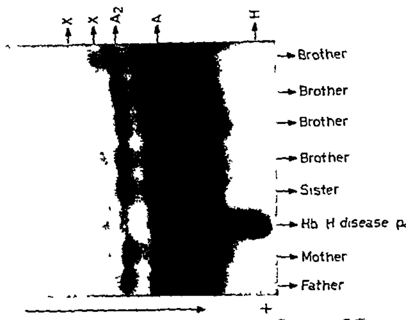


Fig 2 Starch gel electrophoresis in discontinuous tris-citrate boric acid buffer H 9.5 showing the Hb pattern of a Hb H disease patient with X components, and ie same X components in his mother and two brothers Benzidine stain

ty (table I, fig 2) In this type of Hb H disease the condition clearly results from inheriting 2 different abnormal genes, one of which leads to the presence of the abnormal component on electrophoresis at alkaline pH in such concentration that it is easily detectable with special techniques, while the other gene does not give rise to the formation of such an abnormal component, at least it is not demonstrable with available techniques

In 7 patients with Hb H disease (6 of them adults) in 6 families, we could not detect any abnormal X component, *not even in highly* concentrated haemolysates (table II) Unfortunately, both parents of only one of the 7 cases, a child could be examined, and both were without the X component (family No 1a) Two other patients in this group were sisters whose mother did not have the X component either and whose father was dead (family No 2a) The 4 examined children of a fourth patient had only haemoglobins A and A_2 (family No 3a) The fifth patient

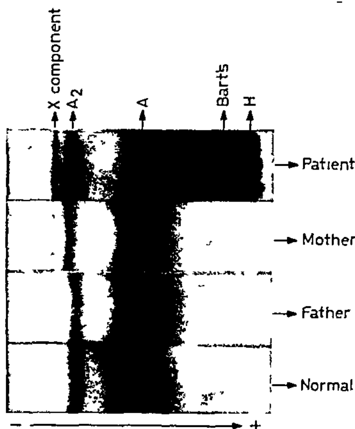


Fig 1 Cellulose acetate electrophoresis in tris EDTA boric acid buffer pH 8 9, showing the Hb pattern of a typical Hb H disease patient with an X component, his mother's with the same component and his father's without that component compared with a normal control O dianisidine stain

ined, one parent of each subject studied invariably carried the same small slow-moving component in the blood, while the other did not (fig 1) One case (family No 7) at first seemed to be exceptional because, although the patient had the abnormal X component, neither parent did However, blood group studies revealed that the man believed to be the father of the patient could not have been The propositus had blood group A, the mother blood group O, and the father blood group B After close inquiry the mother admitted that the child was adopted after birth, but she did not want this to be known In families where other members were available for study in addition to the parent having the abnormal Hb component, several siblings also carried this abnormal-

Table II Hb H disease without X component

Family No	Parents	race	Hb patterns	Siblings Hb patterns A+H[1]	A+A_2	A+A_2+X
1a	father	Malay	A+A_2	1 ♂ 6 years (prop)	1	–
	mother	Malay	A+A_2			
2a	father	Chinese	?	2 ♀ 29 years	–	
	mother	Chinese	A+A_2	♀ 16 years (prop)		
3a	father	Eurasian (Singhalese)	A+A_2	–	4	–
	mother	Chinese	*A+H* (prop)			
4a	father	Malayan Aborigine	?	–	3 +*1* newborn with Hb Bart's (F+A+Bart's)	–
	mother	Aborigine	A+H (prop)			
5a	This family is listed as family No 5 in table I because, although propositus has Hb H disease without X component, several of her children had Hb H disease with X component received from her husband					
6a	father	Chinese	A+A_2	–	1 newborn with Hb Bart s (F+A+Bart's)	
	mother	Chinese	*A+H*			

[1] The Hb H disease patients had varying amounts of Hb Bart's, Hb A_2 and Hb F, sometimes the amount is so low that they could not, or almost not, be detected It also depends upon the buffer used how clearly these components were demonstrated on starch gel electrophoresis

in this group (family No 4a) was a Malayan aborigine who had 4 children One of them, a newborn, had a small amount of Hb Bart's while the 3 older children had a normal haemoglobin pattern The sixth patient was of special interest in that her husband carried the abnormal X component Of the 5 children resulting from their marriage, 2 had Hb H disease with the X component, 2 had normal haemoglobin pattern without the component, while the fifth had the X component without the disease This family is listed in table I (family No 5) because 2 children had Hb H disease with the X component Another interesting feature in

Table I Hb H disease with X component

Family No	Parents	race	Hb patterns	Siblings Hb patterns A+H+X[1]	A+A_2	A+A_2+X
1	father	Chinese	A+A_2	2	4	4
				♀ 18 years		
	mother	Chinese	A+A_2+X	♀ 11 years (prop)		
2	father	Malay	A+A_2+X	1	3	5
				♀ 17 years (prop)		
	mother	Malay	A+A_2			
3	father	Chinese	A+A_2	1	–	–
	mother	Chinese	A+A_2+X			
4	father	Malay	A+A_2+X	–	3	3
	1st mother	Malay	?	–	–	–
	2nd mother	Malay	A+A_2	1	5	–
				♂ 1½ years (prop)		
5	father	Malay	A+A_2+X			
	mother	Malay	*A+H* (prop) without X	2	2	1
				♀ 19 years		
				♂ 11 years		
6	father	Chinese	A+A_2	1	1	1
				♂ 8 years (prop)		
	mother	Chinese	A+A_2+X			
7	father	Chinese	A+A_2	1	–	–
				♂ 10 years (prop) *adopted*		
	mother	Chinese	A+A_2			
8	father	Malay	A+A_2+X	1	–	–
				♂ 13 years (prop)		
	mother	Malay	A+A_2			
9	father	Chinese	?	1[2]	1	1
				♀ 37 years (prop)		
	mother	Chinese	A+A_2			
10	father[3]	Malay	A+A_2	2	3	1
				♀ 14 years (prop)		
	mother	Malay	A+A_2+X	♂ 10 years		
11	father	Malay	A+A_2	–	2	–
	mother[3]	Malay	*A+H+X* (prop)			
12	father	Malay	A+A_2	1	3	2
				♂ 7 years (prop)		
	mother	Malay	A+A_2+X			

[1] The Hb H disease patients had varying amounts of Hb Bart's, Hb A_2 and Hb F, sometimes the amount is so low that they could not, or almost not, be detected It also depends upon the buffer used how clearly these components were demonstrated on starch gel electrophoresis

[2] Propositus had 2 daughters one with Hb H disease with an X component and one with Hb pattern A+A_2+X

[3] Siblings

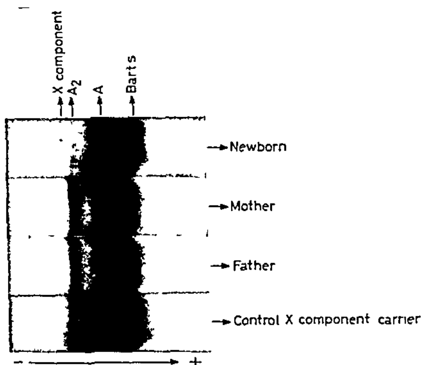


Fig 4 Cellulose acetate electrophoresis in tris-EDTA boric acid buffer pH 8 9 showing Hb pattern of a newborn with Hb Bart s and an X component and those of the parents the father with X component and the mother without O-dianisidine stain

ents were examined None of the 40 parents had the abnormal X component (fig 3) Only one parent of each of 8 other babies could be examined, none had the abnormal X component Two other newborns had the X component in addition to Hb Bart s One had a small amount of Hb H in addition to a 3 8 percent level of Hb Bart s The other, with 3 3% Hb Bart s, was stillborn

The fathers of these 2 newborns had the X component, the mothers did not (fig 4) In addition to that we found one newborn with Hb Bart s level of 22 5% without the X component Unfortunately, the parents refused to be bled Also, the parents of 4 infants with hydrops foe-

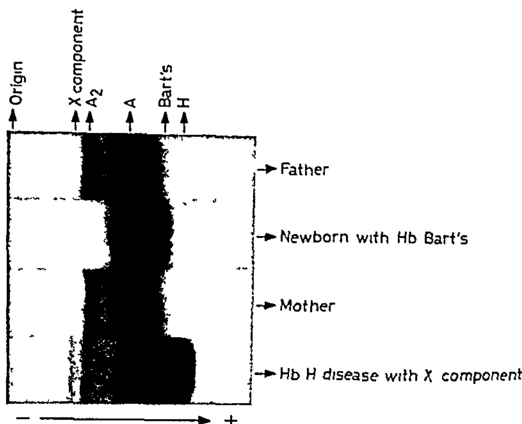


Fig. 3. Cellulose acetate electrophoresis in tris EDTA boric acid buffer pH 8.9 showing Hb patterns of a newborn with Hb Bart's without an X component and of parents also without an X component. O dianisidine stain.

this family is that the husband had also hereditary ovalocytosis and 2 of the children inherited this ovalocytosis gene from the father independently from the X component. The seventh patient in this group (family No. 6a) was the mother of a newborn with Hb Bart's, without X component, her first and only child. She was detected through routine examination of parents of newborns with Bart's haemoglobin. Her husband's haemoglobin pattern was normal.

The X component in relation to Hb Bart's in newborns. To find out the relationship between the X component and Hb Bart's during infancy, babies with this haemoglobin and their parents were examined. Of 20 newborns with Hb Bart's without an X component (10 with Hb Bart's more than 5% and 10 with Hb Bart's less than 3.3%), both par-

several times (fig 1) It is clearly visible in freshly made haemolysate and its presence is, therefore, not due to aging of the haemolysate We also found that the concentration of the abnormal component was high er in patients with Hb H disease than in parents (fig 1) or siblings who had the same abnormal component without Hb H When their very concentrated haemoglobin solutions were run on starch gel electrophoresis as well as on cellulose acetate paper, the abnormal X component could easily be seen with the naked eye as a pink spot or line, even before staining with benzidine or o-dianisidine In dilute concentrations the X component can easily be missed even after staining Further, in patients with Hb H disease we sometimes noticed that in starch gel the amount of Hb A_2 was low or sometimes not detectable and the abnormal X component was relatively high However, when examined on cellulose acetate paper, the amount of haemoglobin component with the mobility of Hb A_2 was relatively higher, the amount of the X component, lower After prolonged staining of starch gels in benzidine or o-dianisidine a second abnormal spot with a more cathodic mobility than the X component appeared in the haemolysates of patients with Hb H disease and in those of their relatives with the X component, so that in fact there were 2 X components They resembled the Gower I and II haemoglobins in mobility One should not, however, confuse these components with the catalase spot visible after staining with benzidine or o-dianisidine, it has a mobility only slightly different from that of the X components at pH 9 5 However, this catalase spot turns green when immersed in Teepol for some time [18]

Discussion

This study revealed that most persons with Hb H disease in West Malaysia have a small slow-moving haemoglobin component in their blood slower than Hb A_2 on electrophoresis at alkaline pH We tentatively called it the X component In every such case one parent, but only one, also had the slow-moving haemoglobin component. Thai workers also detected this slow-moving component in several persons with Hb H disease [21] but did not realize that this abnormality was always inherited from one of the parents, who invariably had the same abnormal component We conclude that Hb H disease in West Malaysia is very often the result of inheriting 2 genes only one of which can be shown with available techniques to lead to the presence of a demonstra-

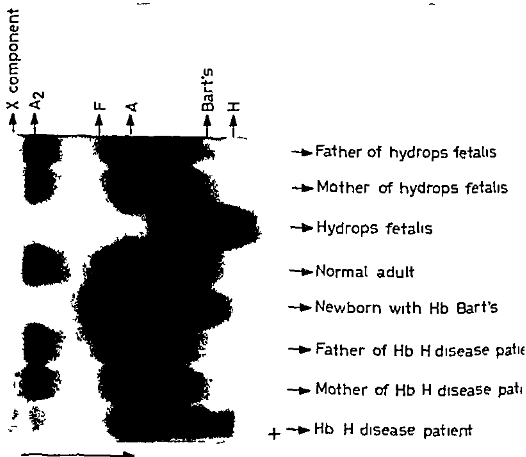


Fig 5 Starch gel electrophoresis in tris EDTA boric acid buffer pH 8 0 showing the Hb patterns of a case of hydrops fetalis and a case of Hb H disease with those of their parents The Hb H disease patient shows an abnormal X component as does one of his parents This component is not seen in the hydrops fetalis patient and in none of her parents Benzidine stain

talis with a large amount of Hb Bart's a condition described earlier [10, 12] were examined, and none had the abnormal X component (fig 5)

Characteristics of the abnormal X component In the standard tris-EDTA-boric acid buffer at pH 8 0 and at pH 8 6 the abnormal component migrated slightly more cathodically than Hb A_2 It was most clearly shown in discontinuous tris-citrate boric acid buffer at pH 9 5 and can also easily be seen on cellulose acetate paper electrophoresis with tris-EDTA boric acid buffer at pH 8 9, especially which had been used

trait carriers with normal haemoglobin pattern and normal persons The above ratio suggest a distribution of more or less $^1/_4$, $^1/_4$ and $^1/_2$ ($^1/_4+^1/_4$) and indicate that the 2 abnormal genes found in the parents are probably alleles

In 7 patients with Hb H disease in 6 families no abnormal small slow-moving X component could be detected, and none of the available relatives had it except several children of one patient This one exception was a patient, married to a man with the abnormal X component, and several of their children carried the same component as the father (family No 5, table I) They apparently did not inherit it from their mother It is possible that cases of Hb H disease without X component have resulted from the combination of the α thal$_1$ and α-thal$_2$ genes, which manifest themselves in the newborn period by the presence of respectively appreciable (5 to 12%) and trace (1 to 3%) amounts of Hb Bart s [14, 21] Therefore, we believe that at least 3 abnormal genes may lead to Hb H disease, a condition resulting when 2 of the 3 combine Perhaps most cases of the disease in Thailand are of the type without the X component However, WASI *et al* [21] observed several Thai patients with the X component, so the first type must also occur in that country A blood sample from a patient with Hb H disease sent by Dr D TODD from Hongkong was also found by one of us (LIE INJO, unpublished) in 1966 to have the X component indicating that this type also occurs in Hongkong Chinese

LEHMANN and CARRELL [9] and LEHMANN [8] put forward a theory on the inheritance of Hb H disease based on the assumption that man has 2 genes for α-chains and one gene for β-chains Thus, one person could possess 1 to 4 α-thalassaemia genes, which would explain the variability in the expression of α thalassaemia KATTAMIS and LEHMANN [7] thought that clinical and haematological findings could be classified by the number of α-genes involved minimal changes when 1 gene is present, more pronounced changes when 2 genes are abnormal, Hb H disease when 3 genes are present, and hydrops foetalis when all 4 genes are abnormal This theory sounds very attractive, especially since BRIMHALL *et al* [1] have presented evidence for the presence of 2 α-chain genes in man. However, several objections have been put forward. One is that no Hb A is present in Hb Q-H disease (Hb Q-α thalassaemia) [3, 11, 13, 19] while one would expect about 25% if there is a duplication of the α-chain gene locus LEHMANN [8] suggested that the Hb Q gene in such cases is linked to an α-thalassaemia gene instead of to a normal α-chain

ble amount of an abnormal slow-moving component Less often the Hb H disease is not associated with a slow-moving component

Hb H disease is currently believed to result from inheriting the so called α-thal$_1$ and α-thal$_2$ genes which, in the newborn period, are manifested by the presence of respectively appreciable and trace amounts of Hb Bart's [20] The finding of NA-NAKORN *et al* [16] that 30 of 31 offspring (almost 100%) of patients with Hb H disease had Hb Bart's either in trace or appreciable amounts, is strong evidence for this assumption If the 2 genes involved in producing Hb H disease were the same as those associated with Hb Bart's in the newborn period, one would expect either the newborns with α-thal$_1$ or those with α-thal$_2$ gene to have one parent with an abnormal X component, because most patients with Hb H disease in this country and one parent of each of these patients have this abnormal component However, the parents of our newborns with Hb Bart's (those with Hb Bart's level above 5% as well as those with level below 3 3%) did not show the X component Thus, we conclude that one of the genes usually associated with Hb H disease in West Malaysia is not associated with Hb Bart's in the neonatal period, and that the other gene is One would, therefore, expect 50% of the offspring of patients with Hb H disease with an X component married to normal persons to carry Hb Bart's in the blood, not 100% In this respect, the findings of WONG [22] in Singapore are more in line with our theory He found that almost 50% of the offspring of his patients with Hb H disease who were married to normal persons had Hb Bart's during the neonatal period It must be said, however, that most of his newborns with Hb Bart's had a high level of this haemoglobin (a mean level of 10% Hb Bart's) They have probably missed those with trace amount of Hb Bart's

In families with Hb H disease associated with the X component (excluding those in which no brother or sister of a patient with the disease was available for study), 43 siblings resulted from the marriage of a carrier of X component and a carrier of α-thalassaemia with normal haemoglobin pattern Families 5 and 11 are ommitted for evaluation because the mother has Hb H disease In family 4 only the marriage with the second wife was considered since the marriage with the first wife did not produce a Hb H disease patient In family 9 the father is assumed to have the X component Nine (20 9%) of them had Hb H disease, 14 (32 6%) had the X component while 20 (46 5%) had normal haemoglobin pattern The last group consists presumably of α-thalassaemia

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gene, so that the Hb Q is always inherited with the α thalassaemia gene, thus depressing the α-chain production Our findings provide a more simple explanation for the variability of Hb H disease, although they need not be in disagreement with the theory of α chain gene duplication They indicate the existence of 3 abnormal genes which may lead to the disease, which results when 2 of the 3 combine Severity of clinical and haematological symptoms then depends upon which type of abnormal gene is present and upon which types of the 3 abnormal genes are involved in any particular combination Haematological findings in the different conditions will be discussed in another paper

In our study of newborns, two babies with Hb Bart s (3 3% in one, 3 8% in the other) with the X component, each had 1 parent carrying the same abnormal X component, it was absent in both parents of 20 other newborns with Hb Bart's without the X component This indicates that the 2 newborns had 2 abnormal genes, one associated with Hb Bart's in the newborn period and the other with the X component One newborn had Hb Bart s level of 22 5% without the X component These 3 babies probably represent Hb H disease in the newborn period They are, however, not listed in table I

The finding of Hb H disease in 2 Malayan aborigines, one newborn and one adult, shows that α-thalassaemia occurs in this people, including the gene associated with the X component

Acknowledgements We wish to thank all colleagues for sending patients for study, the staff of the labour room of the Maternity Hospital Kuala Lumpur, for collecting cord blood samples and our laboratory technicians in the Blood Genetics Section and the Haematology Division of the Institute for Medical Research for their valuable technical help

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Note added in proof A short report on the findings described in this paper appeared also in the 1970 Annual Report of the ICMRT (International Center for Medical Research and Training), University of California, San Francisco (USA) and was presented at the Second Meeting of the Asian Pacific Division of the International Society of Haematology, Melbourne (Australia) May 1971

Since the completion of this paper, the authors have examined other families with Hb H disease giving similar results as described in this paper

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Acta haemat 46 121-128 (1971)

Cytology of Tuberculin Reaction[1]

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Abstract The cytology of tuberculin reaction as an example of delayed hypersensitivity was investigated by using the skin window technic The inflammatory reaction to purified protein derivative (PPD) in sensitized subjects did not differ essentially from that in non sensitized subjects The data show that the cytology of delayed hypersensitivity reaction consists of an ordinary inflammatory reaction during the first 24 h

Key Words
Inflammation cytology
Skin window technique
Tuberculin reaction

The histology of delayed hypersensitivity reactions has been a subject of debate for several decades In 1932 DIENES and MALLORY [2] put forward the view that the tuberculin reaction consisted primarily of mono nuclear cell infiltration with only secondary exudation of polymorphs This view has been widely accepted although there have been several contradictory reports FOLLIS [3] stressed the similarities of the cellular response of tuberculin reaction with ordinary inflammatory reactions REBUCK and YEATS [7] reported marked differences in the leukocytic cycles in the cutaneous tuberculin reactions between sensitized and non-sensitized subjects The investigators found defective and retarded mononuclear responses due to depression of lymphocytic participation at various stages in the tuberculin reaction

SPECTOR [10] believed that the controversy concerning the histology of delayed hypersensitivity reactions was caused by the examination of the lesions at different times after the challenging dose Those investigators who sacrificed the experimental animals during the first 2–3 h re-

[1] A preliminary report was presented at the 5th Congress of the Asian and Pacific Society of Haematology Istanbul September 1969

ported mainly polymorphonuclear emigration Others found predominanlty lymphocytic and monocytic cellular reaction when they sacrificed the animals at later hours BOUGHTON and SPECTOR [1] re investigated the problem by examining large numbers of animals at different times after the challenging dose In guinea pigs the tuberculin reaction showed a biphasic pattern, having a minor peak at 3–4 h predominantly of polymorphs followed by a mononuclear invasion at 5–6 h, and a major peak at 8–12 h again composed of polymorphs These authors concluded that the histological appearance of delayed hypersensitivity reactions consisted essentially of an inflammatory reaction of exaggerated intensity but delayed onset In the light of their findings we re investigated the problem by using the skin window technic of REBUCK et al [6, 7, 8] on large numbers of cases having positive and negative tuberculin reactions

Methods and Material

The skin window technich of REBUCK et al [6 7 8] was used with slight modifications An area of 0 5–1 0 cm in diameter on the volar surface of the forearm was denuded of epithelium by scraping with a scalpel The papillary layer of the corium was reached as indicated by the appearance of fine bleeding points The

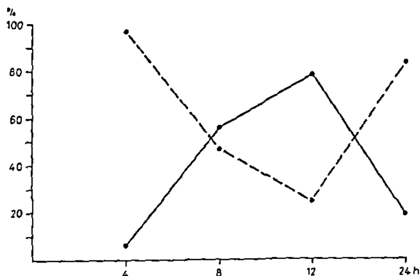


Fig 1 The composition of the cellular reaction in the first 24 h in PPD positive subjects (the figures represent the mean of 24 subjects) ● - - - ● Polymorphs ●——● Mononuclears



Fig 2 Polymorphonuclear leukocytic reaction in a PPD-positive subject at 4 h Rare mononuclear cells are also seen

Fig 3 A mixture of mononuclears and polymorphs at 8 h in a PPD positive subject

Fig 4 Mononuclear cells predominating at 12 h in a PPD-positive subject.

lesion was challenged at zero hour with 0 15 ml of purified protein derivative (PPD)[1] first strength. The lesion was then covered with a sterile slide and fixed to the skin with surgical adhesive tape The cells of the inflammatory exudate migrated to the undersurface of the slide The slides were changed at 4 6 8, 10 12 14 and 24 h In 3 subjects the lesions were studied also at 48 72 and 120 h In 7 cases the skin windows were performed 72 h after the application of PPD and followed for 24 h

[1] PPD was prepared at the Refik Saydam Institute Ankara

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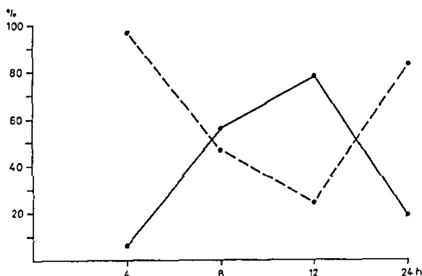


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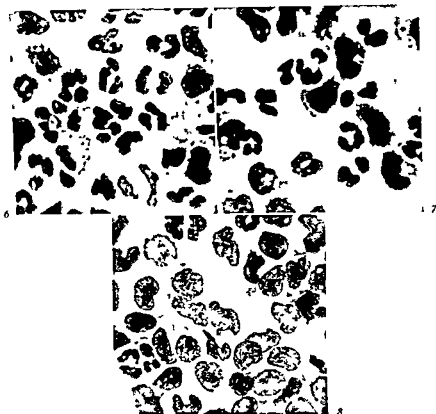


Fig 6 Polymorphonuclear leukocytic reaction in a PPD-negative subject at 4 h A few mononuclear cells are also seen.

Fig 7 A mixture of mononuclears and polymorphs at 8 h in a PPD negative subject

Fig 8 Mononuclear cells predominating at 12 h in a PPD-negative subject

b) Two PPD positive subjects had an inflammatory response com posed mainly of polymorphs from 4 through 24 h Small numbers of mononuclear cells usually appeared around 8–12 h The overall cellular ity was numerically inferior to that of group 1a A second skin window performed on the opposite arm revealed similar findings in both sub- jects

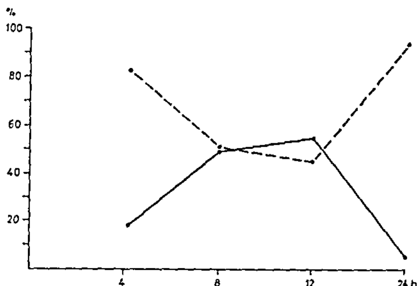


Fig 5 The composition of the cellular reaction in the first 24 h in PPD-negative subjects (the figures represent the mean of 16 subjects) ●----● Polymorphs ●——● Mononuclears

Fifty lesions on 47 individuals were studied 26 of the 47 subjects studied had positive skin tests to PPD Twenty one individuals were PPD negative Of the 26 PPD positive individuals 7 had active tuberculosis in either the form of tuberculous adenitis or of tuberculous pleuresy The remaining 26 individuals were in the hospital with unrelated illnesses Three of the 21 PPD negative individuals were subsequently diagnosed as Hodgkin s disease These are presented separately The remaining 18 PPD negative subjects were mostly patients from the country, hospitalized with various unrelated illnesses

Results

1 Tuberculin-Positive Group (33 Subjects)

a) Twenty-four PPD positive subjects showed an intense, acute inflammatory response characterized by mainly polymorphonuclear infiltration at 4–6 h followed by mononuclear cell infiltration from 8 to 14 h The mononuclear cells were mainly macrophages with spongy, irregular cytoplasm and large indented, folded or oval nucleus A few large lymphocytes and monocytes were also present A new invasion of polymorphonuclear leukocytes were seen at 24 h (fig 1–4) In 3 PPD positive subjects who were followed for 120 h cocci appeared at 48 h along with polymorphs and mononuclears

derived from the circulating small lymphocytes which go through a transformation to become macrophages SPECTOR [10] stated that almost all the mononuclear cells in the inflammatory exudate were derived from circulating monocytes This contraversy concerns not only delayed hypersensitivity reactions but inflammatory responses of all types and is beyond the scope of this investigation

We found that the skin window technic was not suitable for the investigation of the tuberculin reaction longer than 24 h The presence of secondary infection in the lesion as indicated by the appearance of cocci on the slides interfered with the original cycle Polymorphonuclear leukocytic reaction in response to infection was seen in the 48-, 72- and 120 hour preparations

The intensity of the inflammatory reaction seemed to differ slightly from one subject to another in both groups The reasons for the individual differences in the inflammatory response to an exciting antigen was not further investigated Two of the 26 PPD positive subjects (8%) and 5 of 21 PPD negative subjects (24%) showed defective inflammatory responses The 3 Hodgkin's cases confirmed our previous finding that the inflammatory cycle as followed by the skin window technic is abnormal in this disease When these cases are excluded, the percentage of cases with defective inflammatory response falls to 11% in the PPD negative group There was no correlation between the degree of tuberculin positivity and the intensity of the inflammatory reaction in the sensitized subjects

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2. Tuberculin-Negative Group (21 Subjects)

a) Sixteen of the 21 subjects showed an intense, acute inflammatory response similar to those shown by the tuberculin-positive group The polymorphonuclears appeared at 4 h and dominated the picture by 8 h Mononuclear cells were replaced by a new wave of polymorphs by 24 h (fig 5–8)

b) Two of the 21 PPD-negative subjects had an inflammatory response composed mainly of polymorphs in all the preparations from 4 to 24 h Mononuclear cells appeared on 8–12 h preparations in small numbers The overall cellularity was numerically inferior to those of groups 1a and 2a

c) Three PPD-negative subjects with biopsy-proved Hodgkin's disease also showed a defective inflammatory response composed mainly of polymorphs and a few scattered mononuclears

Discussion

In a previous publication [4] we have shown the chain of cytological events in the simple cycle of acute inflammation in man, when the exciting antigen is a non-pyogenic one to which the subject is not systemically immunized The migration of polymorphs was prominent from 4 to 6 h followed by a mononuclear cell invasion from 8 to 14 h In the 24-hour preparations there was a new wave of polymorphonuclear leukocytes *The inflammatory cycle was found to be abnormal in leukemias,* lymphomas and aplastic anemias

When the exciting antigen is one to which the subject is systemically immunized, then this reaction may be considered an example of delayed hypersensitivity The data obtained by studying 33 PPD-positive and 21 PPD-negative individuals revealed no essential differences between these 2 groups in the chain of cytological events following the application of *the tuberculin antigen The polymorphonuclear and the mononuclear in-*filtrations were similar in morphology and timing during the first 24 h of the tuberculin reaction Our findings are in accord with the view that the presence of mononuclear cells is not confined to hypersensitivity states They are part of the inflammatory cycle in non-sensitized individuals [4] and in the acute inflammation caused by other agents [5] The nature and the origin of the mononuclear cells found in an acute inflammation *is still a matter of debate* REBUCK *et al* [6, 7, 8] believed them to be

derived from the circulating small lymphocytes which go through a transformation to become macrophages SPECTOR [10] stated that almost all the mononuclear cells in the inflammatory exudate were derived from circulating monocytes This contraversy concerns not only delayed hypersensitivity reactions but inflammatory responses of all types and is beyond the scope of this investigation

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References

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granulocytes was quite negligible Evidence that radioactivity in circulating blood was due primarily to labelled lymphocytes was obtained during the first experiment by separating leukocytes, erythrocytes and platelets from circulating blood samples by sedimentation, differential centrifugation and hypotonic lysis Evaluation of the radioactivity in the different fractions showed that the largest portion of the radioactivity was in the leukocyte fraction, whereas only a minimal part of the label was associated with erythrocytes and platelets

Circulating radioactivity showed a strictly similar pattern in all cases examined A large portion of the labelled cells infused disappeared rapidly from the circulation, and after 15 min only 20–25% of the total amount of label injected was still detectable in the circulating blood Within 48 h the values of circulating radioactivity decreased to about half the figures found at 15 min, but in the following week the circulating labelled cells decreased very slowly (fig 1) The disappearance curve had 2 components, a first one with an average half disappearance time of about 18 h, and a second one with a half disappearance time of about 7 days

The pattern of variations in organ radioactivity, over a period of 24 h, is shown in figure 2 Heart and lung showed a strictly parallel be-

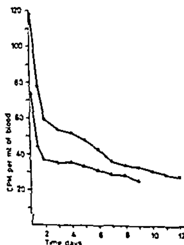


Fig 1 Disappearance curves of labelled lymphocytes from blood of 2 subjects.

of chronic lymphocytic leukemia using ^{51}Cr They maintain that the kinetic pattern in this disease is quite similar to that observed in normal individuals, although the results obtained in the latter have not been reported

In the present study, normal circulating lymphocytes, separated as accurately as possible from other blood cells, were labelled with radioactive chromium, and reinfused Changes in circulating radioactivity, as well as in surface radioactivity over spleen, liver, heart and lung were recorded over a period of several days

Materials and Methods

Our investigations were performed in 4 hematologically subjects in age from 34 to 47 years 900 ml of blood were drawn from each patient into large cylinders where 150 ml of dextran 6% and 30 000 U of heparin had been previously added The initial volume of blood was then restored by transfusing an equal amount of fresh blood Erythrocytes were allowed to sediment at 37 °C until 450–500 ml of supernatant were obtained This was then diluted with an equal volume of balanced salt solution prewarmed at 37 °C, and filtered through a column of commercial nylon fibers (Leukopak Fenwall) The filtered cell suspension was then centrifuged for 20 min at 800 rpm in a refrigerated centrifuge (Model PR2 International Equipment Co Boston Mass) in order to precipitate lymphocytes while leaving in suspension most of the platelets The sediment was resuspended in hypotonic solution to obtain the lysis of erythrocytes and this procedure was repeated 3 times After the last centrifugation lymphocytes were resuspended in autologous plasma at 37 °C The final yield of the separation procedure was about 1×10^{9} lymphocytes These cells were incubated with 300 μc of ^{51}Cr ($Na_2{}^{51}CrO_4$ spec act 100–300 mc/mg) for 45 min at 37 °C and the cells were washed twice with autologous plasma A small aliquot was separated for cell counting and evaluation of specific activity, and the remaining cells were infused rapidly into the patient who had been already prepared for simultaneous scanning on lung heart liver and spleen Body surface scanning was performed with 4 2 in uncollimated thallium activated sodium iodide cristals and a γ spectrometer At various intervals over a period of 10 days determinations were made over areas marked with a skin pencil and keeping the subjects exactly in the same position Following infusion of the labelled cells 10-ml blood samples were obtained after 15 and 30 min 12 and 24 h and once daily thereafter for periods up to 10 days

Results

The infused cell suspensions contained about 1×10^{5} lymphocytes $1–2\times10^{4}$ erythrocytes and $1–3\times10^{4}$ platelets per μl Contamination by

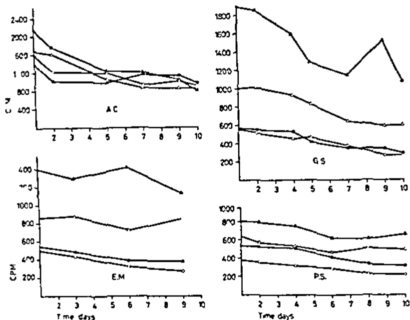


Fig 3 Changes in body surface counting from the 2nd to the 10th day after the infusion of labelled lymphocytes in 4 subjects ● Hearth ○ lung △ liver ▲ spleen

tory answers. The mechanisms by which cells become labelled with chromium remains uncertain as well as the degree of chromate toxicity to the cells and the real amount of elution and reutilization of the label However data so far reported by DRESCH and NAJEAN [2] McMILLAN and SCOTT [7] SPIVAK and PERRY [10] suggest that neither toxicity nor elution or reutilization represent a major problem to the use of chromate as a label for lymphocytes The changes observed in circulating and organ radioactivity are therefore presumably reflecting real changes in the distribution of normally behaving cells

In all our experiments a large portion of the labelled cells apparently disappeared from the circulating blood within a few minutes after the infusion Since no early accumulation of radioactivity was seen during this period in lungs liver or spleen the apparent loss may be explained as suggested by SPIVAK and PERRY [10] by dilution of the labelled lymphocytes in an intravascular pool of cells much larger than that calculated from the blood volume and the blood lymphocyte count If this explana

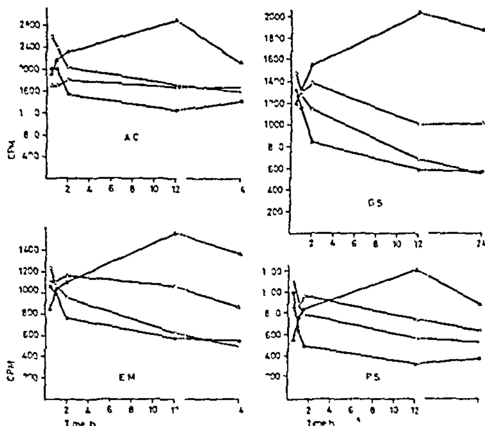


Fig 2 Changes in body surface counting during the first 24 h after the infusion of labelled lymphocytes in 4 subjects ● Hearth ○ lung △ liver ▲ spleen

haviour thus indicating that no sequestration of the labelled cells took place in the latter An early increase of radioactivity was observed over the liver but after 2 h no further rise was found On the contrary labelled cells continued to accumulate in the spleen during a period of at least 12 h so that radioactivity values were found higher about twice the initial values A gradual decrease began in all cases between 12 and 24 h and continued over a period of 6–8 days (fig 3) In one case a temporary rise of spleen radioactivity was again observed after 9 days

Discussion

Many questions concerning the suitability of the ^{51}Cr labelling technique for studying leukocyte kinetics have not received so far satisfac

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tion is correct, the 'marginating' pool of lymphocytes in our cases was from 3 to 4 times larger than the circulating pool

The disappearance from the blood, during the first 24 h, of a relevant portion of the circulating radioactive cells remaining after the initial equilibration is presumably related to their accumulation in the spleen In fact, the pattern of surface radioactivity clearly suggests that the labelled cells pass rapidly through lungs and also liver, whose radioactivity remains proportional to blood radioactivity, whereas part of the labelled cells leave the vascular bed in the spleen, thus increasing the radioactivity over that organ This explanation is in keeping with the results of experiments in rats showing that labelled small lymphocytes some hours after the infusion are located primarily in the white pulp of the spleen [3, 5] However, splenic accumulation does not continue, presumably because equilibration occurs, after 12–24 h, between intravascular and extravascular pool of splenic lymphocytes, and the latter are then gradually released from the spleen Since no further rise is observed in circulating radioactivity, the released labelled cells apparently equilibrate with a larger extravascular pool, presumably including the lymphocytes of lymph nodes and whole body tissues

The pattern of normal lymphocyte kinetics resulting from our observations confirms what it has been maintained by SPIVAK and PERRY [10], i e that normal and CLL lymphocytes have a similar kinetic behavior However, we were unable to observe the repeated fluctuations in splenic activity seen by SPIVAK and PERRY in their leukemic patients This may be due to the much lower amount of cells which are labelled in our hematologically normal subjects, although a real difference in the traffic in and out of the spleen cannot be excluded Much further study and possibly the use of a different label will be necessary to obtain a satisfactory knowledge of the highly complex lymphocyte traffic in human organisms

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Table I Some earlier data on the distribution of breaks in human chromosomes

Authors	Material	Treatment
AYA *et al* [1]	lymphocytes	herpes simplex virus
COHEN [2]	lymphocytes	streptonigrin
COHEN and SHAW [3]	lymphocytes	mitomycin C
GEBHART [4]	lymphocytes	myleran
HAMPEL and BALIO [6]	lymphocytes	X rays
HAMPEL *et al* [8]	lymphocytes	several cytostatic agents
HAMPEL and LEVAN [9]	fetal lung cells	*low temperature*
HAMPEL *et al* [10]	lymphocytes	activated cyclophosphamide
HAMPEL *et al* [12]	lymphocytes	dimethylbenzanthracene
KELLER and NORDÉN [13]	lymphocytes and bone marrow cells	patients with B_{12}-deficiency
KERKIS *et al* [14]	lymphocytes	patients with acute viral hepatitis
KIHLMAN *et al* [15]	lymphocytes	deoxyadenosine, cytosinearabinoside
KRONE *et al* [16]	fibroblasts	hydroxylamine, bromodeoxyuridine
LUBS and SAMUELSON [18]	lymphocytes	untreated
NICHOLS *et al* [20]	lymphocytes	deoxyadenosine, cytosinearabinoside, morbilli virus
STOPIK and HAMPEL [21]	lymphocytes	N substituted cyclophosphamide derivatives

total time of incubation was 72 h at a temperature of 37 0±0.2 °C. These samples were exposed to different chromosome breaking agents 24 h before fixation [5]

The following agents were included in this study (1) 2,3,5-tris-ethylene imino-benzochinone (I 4) (trenimon), (2) 2,4 6-triethyleneimino-1,3,5-triazine (TEM), (3) N,N N triethylenethiophosphoamide (TESPA), (4) derivates of N,N bis-(2 chloroethyl)-N ,O-propylene phosphoric acid ester diamide (cyclophosphamide cytoxan) gained by incubation with rat liver slices [11], (5) N,N bis-(2-chloroethyl)-O-(3-amino-propyl)-phosphoric acid amide ester (A 2), (6) N,N,N-tris-(2-chloroeth [illegible]

distribution of spontaneous breaks was studied in the cells of a patient with glutathione reductase deficiency (NAD [P] H glutathione oxidoreductase, EC 1 6 4.2) [10]

Chromatid and isochromatid breaks were collected from metaphases without visible translocations. Gaps and acentric *fragments were not included in this* study The breaks were visually enumerated in the different chromosomes or chromosome groups 1 2, 3 4-5, 6-12, X, 13-15, 16, 17-18, 19-20 21-22, and Y

Acta haemat 46 136–141 (1971)

Intrachromosomal Distribution of Spontaneous and Induced Breaks in Human Lymphocytes

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Abstract Spontaneous and induced breaks in the chromosomes of human lymphocytes *in vitro* are nonrandomly distributed If one puts several alkylating agents and 5 fluorodeoxyuridine (FUDR) in one group and if one compares the pattern of the distribution of the collected breaks with the findings in another group formed by dimethylbenzanthracene, X rays and spontaneous breaks, the probability was far below 0 005, that the breaks in these two groups were distributed homogeneously within the chromosomal material This indicates the presence of at least two different mechanisms in the origin of chromosomal breakage The findings in the distributions of breaks induced by alkylating agents and FUDR, however, show that the distribution studies related here are no practicable cytological criteria for the detection of different breakage mechanisms

Key Words
Chromosome breakage
Cytostatics
Karyotype
Lymphocytes

Many studies have shown that the distribution of breaks in human chromosomes is nonrandom Table I includes some of these findings

The present study was designed for a statistical analysis of results which were obtained in earlier experiments with X-rays [6] and some cytostatic agents [5, 7, 8, 11, 21] The breaks following the treatment of the lymphocyte cultures with a carcinogenic hydrocarbon (7,12-dimethylbenzanthracene) [12] and the spontaneous aberrations in the cells of a proband with glutathione reductase deficiency [10] were collected from recent cultures

Material and Methods

One hundred and twenty six lymphocyte cultures were prepared and treated according to the method of MOORHEAD *et al* [9] with slight modifications The

Table III List of $\Sigma\chi^2$- values from earlier distribution studies

	$\Sigma\chi^2$ df=41	
Group I		
Trenimon	493 97	a,b $\bar{x}_{1\,2}$ [illegible]
TEM	536 00	(values from all
Thio-tepa	734.31	chromosome segments
		in group I and II,
Cytoxan metabolites	616 49	respectively)
A2 } cytoxan derivatives	567 99	b a = ξ = 1
Z4828 } cytoxan derivatives	464 99	a<b
Z4942 } cytoxan derivatives	436 88	
FUDR	826 45	
		df=41
Group II		$\hat{\chi}^2$ = 72.91
Dimethylbenzanthracene	220 19	$p<0\,005$
X irradiation	202.43	
Spontaneous breaks (glutathione reductase deficiency)	288 64	

One may compare the $\Sigma\chi^2$-values of the cytostatic agents placed in group I with the corresponding figures of group II containing one carcinogen, X-rays and spontaneous breaks (table III) The $\Sigma\chi^2$-values in group II were obviously lower than in group I The next step was to test if the distribution of breaks in group I and II are homogeneous or not

If the average number of breaks counted in the 42 chromosome segments in group I and II, respectively, are represented by the symbols a and b a being less than b, the null hypothesis was b/a equal to ξ equal to 1 With a probability at a level of less than 0 005 this hypothesis could be rejected, too

Discussion

In other words, the inhomogeneous intrachromosomal distribution of breaks in the 2 groups formed by several mutagenic agents indicates at least the presence of 2 different mechanisms in the origin of chromosomal breaks It should be emphasized that the analyzed carcinogen and

For the study of the intrachromosomal distribution of breaks, the long arms and short arms, where possible, were divided into 3 segments equal in size *proximal*, *intermediate*, and *distal* from the centromere If an arbitrary value of 1,000 units is taken for the total length *of the chromosomes of a female or of a male* karyotype the relative lengths of the chromosomal segments can be expressed in parts from 1,000 They were calculated from the data of LEVAN and NICHOLS [17] which are based on measurements of chromosomes in male and female karyotypes

Results

Table II shows an abbreviated example for a distribution of dimethylbenzanthracene-induced breaks *The first column contains the symbols for some chromosome segments* A sample of 1,000 breaks was collected If one assumes a random distribution of the breaks caused by this agent the values in column 2 and 3 must be equal However, this null hypothesis of a mere random intrachromosomal distribution could here be abandoned with a probability of far below 0 001.

Table II χ^2 test for randomness in distribution of breaks induced by 7,12-dimethyl benzanthracene in human chromosomes (abbreviated)

Chromosome segments			Relative length = expected breaks	Found breaks	χ^2
1	l	d	15.25	30	14 26
		i	15 25	12	0 69
		p	15.25	21	2 16
	s	d	13 84	7	3 38
		i	13 84	9	1 69
		p	13 84	7	3 38
2 l		d	16 57	32	14 36
21-22 Y	l		34 14	15	10 73
	s		8 77	–	–
			1,000 00	1,000	$\Sigma\chi^2$ 220 19 df = 41 p < 0 001

l = long arms, s = short arms, d = distal segment, i = intermediate segment, p = proximal segment.

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Acta haemat 46 142–148 (1971)

Pentose Phosphate Metabolism of Erythrocytes in Hepatic Porphyrias[1]

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Key Words
Erythrocyte metabolism
Pentose phosphate shunt
Porphyria hepatica
Transketolase

Abstract The erythrocyte TKA of 13 patients with hepatic porphyria was examined Four showed definitely low enzyme activity, and in another 5 the TKA was close to the lower limit of the normal range In 1 patient the TKA was observed during the manifestation of clinical symptoms and during convalescence In 10 months his TKA graduallv climbed to low normal range Two relatives of this patient with porphyria showed pathologically low erythrocyte TKA values

Fasting can provoke symptoms of acute intermittent porphyria [4] Experimental evidence indicates that the mechanism is probably the induction of δ aminolevulinic acid synthetase in the liver by caloric deprivation Rat experiments have previously led to similar results [3] Allyl-isopropyl-acetamide induced increasing symptoms of porphyria in rats fed on diets with restricted carbohydrate and protein content. But especially in a patient with latent intermittent porphyria did a carbohydrate and protein restricted diet produce an increase in the excretion of δ-aminolevulinic acid and prophobilinogen into the urine [14, 15] Ridley [11] carried out pyruvate test on patients with acute intermittent porphyria with neuropathy. In 6 of 7 patients pathological quantities of pyruvic acid accumulated in the blood after the administration of glucose

In 2 materials, one of 12 and the other 19 patients, the carbohydrate metabolizing enzymes in the red cells revealed that G 6 PD 6-PGD and glutathion reductase activities in hepatic porphyrias were elevated [3, 10]

The present study was concerned with the transketolase activity (TKA) of erythrocytes in patients with hepatic porphyria

[1] Grant from The National Research Council For Medical Sciences, Finland

Material and Methods

The series is presented in tables I, III and IV. A total of 13 patients suffered from porphyrias of different types, and 2 healthy daughters of a man with porphyria were included in the study (table IV) The control series consisted of 278 healthy subjects.

All the patients with hepatic porphyria were examined at the university medical clinic The diagnosis of porphyria, and the determination of its type, were based on medical history, especially the familial history, a careful clinical examination including laboratory tests, roentgenography and the determination of porphyrin metabolites in the urine, faeces and erythrocytes (table I) This part of the study was the responsibility of the team expert on porphyrin disorders (PΚ)

The control series consisted of patients under examination at the university medical clinic They were under observation for some minor symptom, and de-

Table I Presentation of the series examined

No	Sex	Age	Diagnosis	ALA (3.7)	PBG (3.4)	UCP (150)	UUP (30)	EPP (30)	FPP	FCP
									–(100)–	
1	m	45	PAI sympt	19.4	8	285	3 670	32	173	46
2	m	44	PAI, sympt	35.0	14	615	1,100	29	194	16
3	w	26	PAI, sympt	13.7	83	530	2,110	128	98	19
4	m	58	PAI, sympt	14.0	10	420	590	–	–	–
5	w	22	PAI, sympt	4.8	34	5,750	4 650	–	–	–
6	w	35	PAI no sympt	4.6	14	475	86	79	51	11
7	m	34	PV, latent	6.9	48	700	940	27	333	36
8	w	29	PV, no sympt	1.4	59	690	1,160	53	333	36
9	w	25	PV, latent	3.6	2	70	15	38	155	90
10	w	46	PV, latent	5.3	3	197	54	42	666	206
11	w	23	PV, no sympt.	12.5	15	1 860	300	47	603	397
12	m	58	PCT, sympt.	3.7	2	3,300	30,200	17	539	293
13	m	62	PCT, sympt.	3.9	2	720	3,710	32	175	102

PAI = acute intermittent porphyria, PV = porphyria variegata (European form). PCT = porphyria cutanea tarda. Sympt. = the patient had symptoms at the time the sample was taken No sympt. = the patient had no symptoms at the time the sample was taken Latent = the patient had never had symptoms but according to the biochemical status he was affected with porphyria which also occurred familially ALA = δ-aminolevulinic acid, PBG = urinary porphobilinogen, UCP = urinary coproporphyrin, UUP = urinary uroporphyrin, EPP = free erythrocyte protoporphyrin, FPP = faecal protoporphyrin, FCP = faecal coproporphyrin Underneath the abbreviated column headings, the normal upper limits of excretion or content are given in brackets ALA and PBG mg day UCP and UUP μg day, EPP μg 100 ml cells, FPP + FCP aggregate excretion μg g dry weight

tailed clinical examination revealed no organic disease in any of them. A control sample was taken simultaneously with each sample from a patient with porphyria. Otherwise the control material was supplemented and the red cell TKA determined in the same series as the samples of the patients with porphyria.

The porphyrin metabolites in the urine and faeces [9, 12], the porphyrin metabolite of the red cells [5] and the red cell TKA [8] were determined as described before.

The inhibitory action of porphyrin metabolites *in vitro* in the enzyme reaction was studied in the following way. Coproporphyrin I (standard solution 210 μg/ml), coproporphyrin III (standard solution 200 μg/ml), uroporphyrin I (standard solution 165 μg/ml) and uroporphyrin III (38 μg/ml) were dissolved in a 2% ammoniumhydroxide solution to make the concentrations listed above. 0.5 ml of each standard solution was evaporated dry in the vacuum and the residue was dissolved in 2 ml (uroporphyrin III, in 1 ml) distilled water. The resulting solution was diluted 1:10. This final dilution was used in the TKA determinations so that the standard solution was diluted 1:4–1:40 except for uroporphyrin III which was diluted 1:2–1:20. In addition ammonium hydroxide in the same concentration as in the porphyrin solution was evaporated alone, and the residue obtained was diluted with distilled water in the same way as the porphyrin solutions. The zero solution consisted of deionized distilled water.

The erythrocyte TKA was indicated in units (U) μmol sedoheptulose 7 phosphate/ml cellular mass/30 min 37 °C, ph 7.4.

Results

The erythrocyte TKA of the control series (n=278) ranged from 3.5–6.7 U, mean 4.8 U. 3.5 U must be considered the absolute minimum limit of a normal range, the range 3.5–3.8 is a borderline area open to interpretation.

Table II shows that 5 of 13 patients with porphyria had an erythrocyte TKA level at or below the minimum limit of the normal range. If the borderline area is included, 9 of 13 patients had a lowered or low erythrocyte TKA. Table II shows further that 5 patients with acute intermittent porphyria (PAI) were having an acute attack or clinical symptoms at the moment the sample was taken. The TKA of 4 of these patients was definitely at the pathologically low level, and of 1 it was in the borderline area. The TKA of the patients with no symptoms was wholly within the normal range.

In 3 patients with porphyria variegata (PV) the disease was 'latent', and their TKA levels were normal. Two 'no symptoms' patients showed TKA levels of the borderline area.

Table II Erythrocyte TKA, U ml red blood cells

No	Diagnosis	TKA units
1	PAI sympt	2.7
2	PAI, sympt	2.7
3	PAI, sympt	3.7
4	PAI, sympt	2.6
5	PAI, sympt	2.5
6	PAI no sympt	5.4
7	PV, latent	5.2
8	PV, no sympt	3.8
9	PV, latent	4.1
10	PV, latent	4.1
11	PV, no sympt	3.8
12	PCT, sympt	3.5
13	PCT, sympt	3.7

Patient numbers and abbreviations are those of table I TKA controls (n = 278), mean 4.8 min 3.5 and max 6.7 U

Table III The erythrocyte TKA was observed in the course of and after an attack of porphyria

Patient No 4 Observation time and status		TKA units
On admission to hospital	sympt +	2.6
2 days after adm	sympt +	2.6
3 days after adm	sympt +	2.8
4 days after adm	sympt +	3.0
6 months	sympt –	3.1
8 months	sympt	3.2
10 months	sympt –	3.7

In the 2 patients with porphyria cutanea tarda (PCT), both of whom had symptoms, the TKA was low or within the borderline area

Table III presents the TKA findings on a patient (table I, No 4) under observation during an acute attack of porphyria and followed up for 10 months afterwards As long as the attack lasted the TKA was extremely low After the acute attack was over the patient was seen from

Table IV Erythrocyte TKA of patient No 4 (Table I No 4) with porphyria and his daughter (2) also affected with porphyria as well as that of his other 2 healthy daughters (3, 4)

Member	Age	Observations	TKA units
1 Father	58	PAI sympt	2 6
2 Daughter	22	PAI sympt	2 5
3 Daughter	19	healthy	2 7
4 Daughter	28	healthy	3 3

time to time After 6 months there were no more clinical symptoms (abdominal pain, transient paresis, neuritis), but the porphyrin level in excretions was unrecordable Even after 8 months the TKA was still low, and after 10 months in the borderline area of the normal range However, throughout the period of convalescence a slight trend towards the recovery of TKA was visible

Table IV gives the results of the examination of several family members The father (1) and one daughter (2) had clinical symptoms of PAI at the time when the samples were taken, and their erythrocyte TKA was definitely low Two other daughters (3 and 4) had never had any symptoms, and the porphyrins in their excretions were normal In spite of this, their erythrocyte TKA was pathologically low

Porphyrin metabolites in reaction mixture *in vitro* produced no enzyme inhibition

Discussion

Certain dietary treatments have been shown to have a distinct effect on the hepatic G-6-PD activity Since this enzyme is a kind of key to the pentose phosphate pathway and since the TKA plays a considerable role in this pathway, changes in the activities of these enzymes are often parallel It is known that fasting reduces the G-6-PD activity, and affects TKA in the same way [1] The opposite state, in its turn, increases the activity of these enzymes

Since TKA requires co-carboxylase as a co-factor, it may be assumed and, in fact, it has long since been proved [2] that fasting reduces the TKA activity both in the liver and in erythrocytes Apparently this is the case in certain deficiency states, in alcoholics [8] and in certain geriatric groups [8]

Considering that the activities of G 6-PD and TKA are usually parallel, the present results, at least seemingly, are contradictory to a couple of earlier reports [3, 10] These latter rather indicated that in hepatic porphyria G-6-PD, 6 PGD and glutathione reductase are activated in the patient's erythrocytes

According to the present study, especially in the PAI group during the period of acute symptoms the TKA had fallen dramatically to values seldom seen before in another disease The divergent values were checked by duplicate tests, and the determination series always included healthy controls to indicate the reliability of the method The reproducibility of the results of this determination method is 100 ± 3% [7]

Although the porphyrin metabolites tested in the present study failed to inhibit the TKA reaction *in vitro*, it is nevertheless possible than when natural metabolites, either as such or bound to proteins, are the inhibitors, they behave differently from those now tested This possibility may be suggested by the particularly low TKA values in the course of the attacks of acute intermittent porphyria On the other hand, some other intermediate metabolic products may perhaps inhibit the TKA reaction during this period The erythrocytes used in the determination were washed and other porphyrin metabolites contained in the plasma hardly adhered to their surfaces

It was an interesting observation that in hepatic porphyria in its asymptomatic phase the erythrocyte TKA may be nearly or completely normal Nor is it without interest to find that in a family member of a patient with porphyria the erythrocyte TKA may be pathological although the person concerned has never had any clinical symptoms and although the biochemical porphyrin state is fully normal Future studies should devote attention to the activity of glycolytic enzymes during the acute attack during recuperation and in the patient's various family members

The earlier reports concerning the effect of fasting as a porphyria-provoking factor agree very well with the finding that fasting may 'deteriorate the TKA activity which may already be close to the limits of the normal range Future studies should therefore, determine simultaneously at least the key enzymes in the principal glycolytic shunts and then study the member enzymes of these shunts over a large *spectrum at the* same time This method would provide a clearer overall picture of carbohydrate metabolism in porphyria, and leave less room for possibly contradictory speculations An interesting field of the study in this sense

is also afforded by the latent and asymptomatic members of families with a history of porphyria, and by the possibility that low TKA levels and neuropathy of porphyria may be interrelated

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Acta haemat 46 149 156 (1971)

Is Haemoglobin Gα Philadelphia Linked to α-Thalassaemia?

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Abstract Members of a Jamaican family carrying the α-chain variant haemoglobin Hb Gα Philadelphia have been found also to show red cell abnormalities suggestive of a mild thalassaemia trait Hb Gα Philadelphia differs from most other α-chain variant haemoglobins by being present in unusually high percentage in heterozygotes (Hb A + Hb Gα Philadelphia) It is proposed that the gene for this variant is linked with one for α thalassaemia

Key Words
α thalassaemia
Haemoglobinopathies
Hb Gα Philadelphia

It has been pointed out by FESSAS *et al* [1] that α-chain variants ($\alpha_2^X \beta_2^A$) of human adult haemoglobin ($\alpha_2^A \beta_2^A$) are generally present in a lower proportion than are β-chain variants This has usually been associated with the concept that the former are more 'abnormal', perhaps because of the basic importance of the α-chain for all haemoglobins it has been observed for example that the proportion of a haemoglobin variant in the heterozygote appears to vary with the degree to which it is pathological [2] Nevertheless not all α-chain variants have been found in low proportion notable exceptions being Hb Gα Chinese (Hb Gα Honolulu) and Hb Gα Philadelphia both of which have been reported to form more than 40% of the total in heterozygotes [3]

As more haemoglobin variants became known that had homologous substitutions in the α and β-chains respectively it was noted that the α-chain variants amounted to one half of the corresponding β-chain variant This suggested that whilst the same substitution affected one half of the β-chain genes it acted on only a quarter of the α-chain genes in the

respective heterozygotes, i e that the human α-chain gene was duplicated and individuals had 4 of them, not 2 [4] The fact of this duplication is now certain from the findings in a Hungarian family in which 2 α chain abnormal haemoglobins plus Hb A were present in 2 brothers [5] This leaves the difficulty of explaining why some α chain abnormal haemoglobins are exceptional in being present in a proportion of 40% or more, similar to that seen with β-chain variants

The 4 α-chain gene theory has been extended to include a 4 α thalassaemia gene concept [4] and it has become evident that for this to explain the findings in Hb Q H disease, it is necessary to assume a linkage between the α^Q gene and an α thalassaemia gene [6, 7] Such linkage could perhaps explain the high levels of some α-chain variant haemoglobins, referred to above, the assumption being that these heterozygotes possess one chromosome with an α-thalassaemia plus a variant α chain gene, and one with 2 normal α-genes

An examination of a family with Hb Jα Tongariki failed to support this concept [8] In particular, homozygotes who should carry 2 α-thalassaemia genes seemed to be free from the stigmata of thalassaemia, as far as haemoglobin levels, haematocrit and morphological appearances were concerned However, as the authors themselves state, the diagnosis of α-thalassaemia other than Hb H disease is most difficult to establish, and these investigations were done on blood samples collected several days before Red cell counts and osmotic fragility values were not recorded In one homozygote, the haematocrit was 40% The 2 haemoglobin values given for this subject, 14 8 and 12 2 g/100 ml, would give MCHC values of 37 and 30 5%, respectively above and below the normal range

Subjects and Methods

We have studied a Jamaican family with Hb Gα Philadelphia which was discovered in South London during the course of a survey The index case was child 1 a 10-year-old boy, cellulose acetate electrophoresis at pH 8 9 showed him to be heterozygous for an abnormal haemoglobin similar in its migration to Hb S while the slide sickling test and solubility tests for Hb S were negative As a result permission was sought and obtained to take blood samples from both parents and 3 other siblings

Quantitation of the haemoglobins was carried out at the Abnormal Haemoglobin Unit, where the identity of the variant was confirmed by peptide analysis car

ried out by Dr ANDREW LANG Haematological values were obtained on both parents and all 4 children immediately after collection of blood samples, using a Coulter Model S automatic cell counter, standardised by reference to the '4C' standard supplied by the manufacturers and checked by the use of local control samples Osmotic fragility curves were determined with minimal delay on heparinised blood by standard methods on all except child 1 plasma iron and total iron binding capacity were determined in the mother using an auto-analyser, and alkali resistant haemoglobin was measured by the method of BETKE *et al* [9] Blood films made immediately after collection of the samples were examined independently by Prof J L STAFFORD and Dr R F SHEPPARD, the films together with some not connected with the investigations were presented in a random order and identified only numbers

Results

The abnormal haemoglobin, confirmed as Hb Gα Philadelphia, was found in the mother and in the 4 children, all of whom were heterozygotes (fig 1) Sickling tests had proved negative in every case, and all showed on electrophoresis a slow moving α-chain variant of Hb A_2 The father showed only Hb A as a major haemoglobin, but was also heterozy-

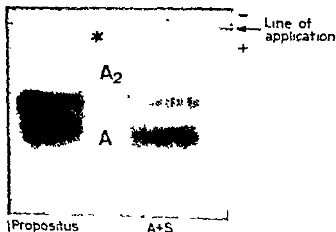


Fig 1 Paper electrophoresis of haemoglobins tris-EDTA borate buffer, pH 8 9 Right sickle trait (Hb A + Hb S) Left haemoglobin of propositus with similar proportion of Hb Gα Philadelphia The star indicates the α-chain variant of Hb A_2 (α_2^G δ_2).

Table I Haematological values obtained for members of the family Samples (a) and (b) were obtained from the mother about 4 weeks apart

	WBC $10^3/\mu l$	RBC $10^6/\mu l$	Hb g/100 ml	PCV %	MCV μm^3	MCH pg	MCHC %
Father	8 0	5 40	15 1	43 6	81	27 8	34 7
Mother (a)	6 9	5 54	13 4	39 9	72	24 2	33 6
Mother (b)	6 6	5 14	13 0	36 2	71	25 3	36 0
Child 1 (10 M)	5 4	6 19	13 1	39 5	64	21 2	33 3
Child 2 (8, F)	8 2	5 15	13 4	37 2	73	26 0	36 1
Child 3 (7, F)	10 2	4 87	12 2	35 1	73	25 1	35 0
Child 4 (6, M)	7 4	5 25	11 1	31 9	62	21 1	34 8

The age and sex of each child is shown in brackets

gous for Hb A_2 and a fast-moving variant of Hb A_2, the latter resembling Hb H_2 Flatbush in its mobility.

The percentage of α^G was 43 5 in the mother This value is based on the amounts of Hb G ($\alpha_2^G\beta_2^A$) and abnormal Hb A_2 ($\alpha_2^G\delta_2^{A2}$), which were 41 9 and 1 6% respectively The children possessed similarly high levels of Hb G*a* Philadelphia, i e around 40%, minor haemoglobin levels could not be relied on as a measure of α^G because of possible confusion by inheritance of the $\delta^{Flatbush}$ gene

The haematological values are presented in table I and the osmotic fragility curves are shown in figure 2 The mother's plasma iron level was 72 μg/100 ml and TIBC was 393 μg/100 ml, levels which do not suggest that iron deficiency was causing any red cell changes The father shows complete normality in all values, the mother and children have normal haemoglobin levels, but despite this they all show slight to moderate reductions in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) The mother, child 3 and child 4 have decreased osmotic fragility, child 2 is borderline in this respect None of the family showed any detectable foetal haemoglobin (Hb F) Comments on the blood films by the 2 observers are listed in table II, The blood film shown in figure 3 is one which shows the abnormalities described, we must stress that in other members of the family these changes were less marked and would not have shown well on a photograph without some selection of fields It may be remarked that the degree of abnormality reported correlates broadly with the osmotic fragility results

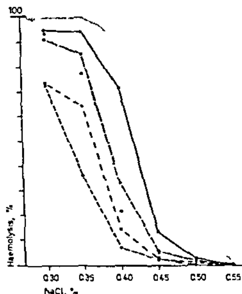


Fig. 2. Osmotic fragility curves of parents and 3 children. Curves of normal controls all fell within the normal range (hatched zone). ●—● Father. ● ● Mother. ●--● Child 2. ●- -● Child 3. ●—● Child 4.

Table II. Comments on the red cell morphology of family members, made independently by 2 observers

Family	Observer 1	Observer 2
Father	normal	slight hypochromia
Mother	slight anisocytosis	slight hypochromia and anisocytosis
Child 1	anisocytosis, occasional target cells	hypochromia, anisocytosis and poikilocytosis
Child 2	normal	normal
Child 3	normal	slight anisocytosis and microcytosis
Child 4	anisocytosis, occasional target cells and spherocytes	hypochromia, anisocytosis and poikilocytosis

Thus all members of this family who carry Hb Gα Philadelphia show some of the features of a thalassaemia trait, although in child 2 these are very slight, being confined to the low MCV and MCH.

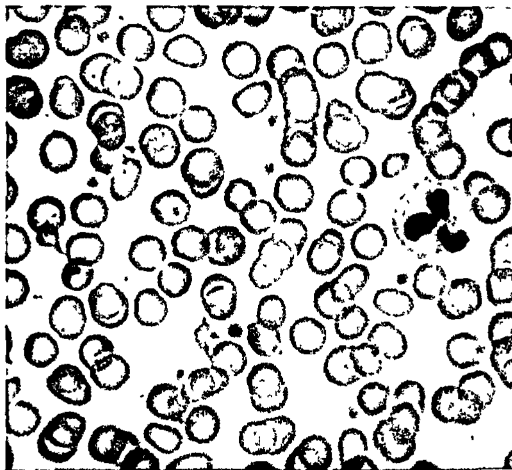


Fig 3 Blood film of child 4, showing red cell changes Jenner Giemsa stain ×400

Discussion

On the basis of the above findings, we are suggesting that in th family the gene for $\alpha^{G\ Philadelphia}$ is linked with an α-thalassaemia gene This provides a possible explanation of a finding not otherwise compati ble with the assumption of 4 α-chain genes in man, namely the repor from Nigeria that the mother of a child with Hb A and Hb Gα possessec Hb Gα only [10] This haemoglobin has not been positively identified a Hb Gα Philadelphia, but all other identified examples of Gα haemoglob ins from West Africa have proved to be of this type, no other Gα var iant has been reported from this region Our suggested interpretation o

this finding is that the gene for $\alpha^{G\ Philadelphia}$ is accompanied on the same chromosome by an α thalassaemia gene Thus *heterozygotes would* have 3 functioning genes (α^A, α^A and α^G) plus 1 α thalassaemia gene, while homozygotes would have 2 functioning α^G genes and 2 α thalassaemia genes the latter producing no α^A peptide chains

The crucial finding in the family reported here is that the children appear to have inherited both a variant α-chain and the stigmata of thalassaemia from their mother We have seen that Hb A₂ and Hb F levels are normal in the mother and may reasonably rule out a coincidental β thalassaemia, therefore a mutant α-gene and an α thalassaemia gene appear to have been transmitted together by the same parent Since other reports of Hb Gα Philadelphia quote high amounts in heterozygotes [3] the duplication theory must imply further that the 2 α-chain loci are closely linked, so as to make crossover an unusual event Were such a crossover to occur, we would predict that it should result in individuals having about 20% of Hb Gα Philadelphia, whose red cells would not show the abnormalities found in our family There are no reasons, on structural grounds why Hb Gα Philadelphia should itself have any pathological effects, since the site of its amino acid substitution, α68 (E17) is at an external, non functional position on the haemoglobin molecule [11]

It would be of interest to re-examine other families with Hb Gα Philadelphia and Hb Gα Chinese for the presence of red cell abnormalities It would be additionally valuable if the opportunity should present itself, to look for haemoglobin Bart s in the cord blood of any newborn heterozygotes in such families

Acknowledgment We are grateful to Dr A C. McReynolds for permission to investigate this family to whose willing co-operation we are indebted

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Acta haemat 46 157–176 (1971)

Zytokinetische Untersuchung einer aleukämischen akuten myeloischen Leukämie

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Abstract Cytokinetics of an aleukemic acute myeloid leukaemia are studied by means of the flash labeling method with ³H thymidine In comparison with normal granulocytopoiesis generation time, phases of the cell cycle and the survival of the blasts in the peripheral blood are prolonged The relative production of cells however, is not altered From the blasts produced in the bone marrow only 1/14 do enter the peripheral blood. This explains very well the aleukaemic course of the disease The results of the blood and the bone marrow obtained by the flash labeling method are compared with 3 mathematical models. Only the model assuming that all proliferating cells enter the G0 phase of the cell cycle after mitosis and that the so-called end cells and G0 cells are released in the peripheral blood is compatible with the results obtained by the *in vivo* flash labeling method

Key Words
Autoradiography
Cell generation
Cytokinetics
Leukaemic cells

Seit die Leukämie 1845 von Rudolf Virchow erstmals als eigenständige Krankheit erkannt wurde, kennzeichnete man das Wesen dieser Krankheit als gesteigerte autonome Vermehrung einer Zellpopulation, deren Differenzierung zu funktionell vollwertigen Zellen gestört ist Das Überhandnehmen der pathologischen Zellart drangte das Postulat einer erhöhten Produktionsrate auf, welche ihrerseits durch eine verkürzte Generationszeit erklärt wurde Diese in neuern Lehrbüchern immer noch vertretene Hypothese bildete auch die Grundlage zu therapeutischen Versuchen

Erst während der letzten zwei Jahrzehnte wurde diese Annahme mit zytokinetischen Methoden überprüft [4, 24] Obwohl aufgrund der neuesten Literatur noch kein in allen Teilen abgerundetes Bild der leukämischen Zellvermehrung herausgearbeitet werden kann drangt sich doch

immer mehr ein Konzept auf, das mit dem klassischen in Konflikt steht. Zytokinetische Untersuchungen zeigten nämlich in der Mehrzahl der Leukämien 1. keine verkürzte, sondern eine normale oder verlängerte Generationszeit der Blasten [2, 6, 8, 15, 17, 23, 25], 2. eine normale oder verminderte Produktionsrate [2, 6, 7, 16, 25], 3. eine deutliche Verlängerung der Aufenthaltszeit der Blasten im Blut [3, 6, 7, 17]. Zudem scheint auch das leukämische Knochenmark neben proliferierenden Blasten eine Population sich nicht teilender Zellen aufzuweisen [2, 8, 16, 17]. Im Unterschied zu diesen kaum mehr zu bezweifelnden Feststellungen lassen sich noch keine sichern Aussagen machen u. a. über die Beeinflussung der normalen Hämatopoese, die Gesetzmässigkeiten des Austritts aus dem Mark und das Zustandekommen aleukämischer Verlaufsformen.

In der vorliegenden Arbeit wurden die zytokinetischen Parameter des Knochenmarkes und des Blutes bei einer aleukämischen myeloischen Leukämie durch *in vivo* Markierung mit ^{3}H-Thymidin bestimmt. Zudem wurde zum ersten Mal anhand mathematischer Modelle die Produktion der Blasten im Knochenmark mit dem Erscheinen im Blut korreliert, um so neue Aussagen über Produktions- und Austrittsmuster zu gewinnen.

Methode

1. Injektion von ^{3}H-Thymidin und Präparation der Autoradiographien. Zur Markierung wurde ^{3}H-Thymidin (0,1 mc/kg Körpergewicht, spez. Aktivität 2,0 mc/mM) verwendet. Nach einmaliger Thymidininjektion folgten unter Lachgasnarkose Sternalpunktionen nach 1, 2, 3, 5, 7, 10, 15, 20, 24, 28, 36, 42, 49 h. Kapillarblutentnahmen nach 1, 2, 3, 4, 5, 6, 7, 10, 12, 15, 18, 20, 24, 28, 36, 42, 49, 52, 63, 72 h. Die angefertigten Ausstriche wurden luftgetrocknet und mit Methylalkohol 15 min fixiert. Nach Überschichtung mit Kodak NTB 2 Filmemulsion und 60tägiger Exposition bei 4 °C wurden die Präparate entwickelt und mit Giemsa-Lösung pH 6,0 gefärbt.

2. Auszählung. Von jedem Knochenmarkausstrich wurden 2000 Blasten und mindestens 50 Mitosen gezählt und deren Körnerzahl und Markierungsindex (% markierte Zellen pro Zellklasse) bestimmt. In den Blutausstrichen erfolgte die Auswertung an Hand von je 3000 Zellen. Die Background-Bestimmung über 1000 nicht markierten Zellen ergab eine durchschnittliche Körnerzahl von 1,5 pro Zelle. In Anwendung der Methode von CLARKSON [6] wurden Zellen mit 8 und mehr Körnern als markiert gewertet. Enthielt anderseits eine Zelle mehr als 100 Körner, so wurden lediglich 100 gezählt. Entsprechend dem Vorschlag von KILLMANN [17] und GAVOSTO [12] wurde versucht, die Blasten nach Grösse und Atypie in Klassen einzuteilen. Dies ergab jedoch wegen der ausgeprägten Polymorphie der z. T. un

vollkommenen Färbung und der unterschiedlichen Ausstrichdicke keine aussagekräftigen Resultate

3 *Bestimmung der Phasen des Teilungszyklus und der Generationszeit* Als Phasen des Teilungszyklus werden unterschieden Mitose (M), postmitotische Ruhephase (G1) DNA Synthesephase (S), prämitotische Ruhephase (G2) Zellen, welche sich aktuell nicht in einem Teilungszyklus befinden jedoch erneut in einen solchen eintreten können werden als G0-Zellen bezeichnet

Die zuverlässigste Methode zur Bestimmung der einzelnen Phasen stellt die Interpretation der Markierungsindexkurve der Mitosen dar, wie sie erstmals von BOND *et al* [5] beschrieben wurde Da zum Zeitpunkt (t_0) der ³H Thymidininjektion ausschliesslich die Zellen der S-Phase markiert werden, entspricht das Zeitintervall bis zum Erscheinen markierter Mitosen G2 die Dauer des ersten Plateaus (50% Anstieg bis 50% Abfall) der S-Phase und das Intervall zwischen dem ersten und dem zweiten Plateau der Generationszeit (t_g) (siehe Abb 1) Zusätzlich wurde die Generationszeit mittels der Halbwertszeit der mittleren Körnerzahl aller sowie der Halbwertzeit der 50% höchstmarkierten Interphaseblasten bestimmt Diese Methode wurde eingehend von CLARKSON diskutiert [6] Sie ergibt obere Grenzwerte Die Mitosezeit wurde anhand der Formel bestimmt Mitosezeit S-Phase = Mitoseindex Markierungsindex [17]

Zahl der Blasten im Blut In den 2 Tagen vor sowie während der Untersuchung sank die Zahl der Leukozyten und der Blasten *in exponentieller* Weise ab Im Hinblick auf den Vergleich mit den mathematischen Modellen war es daher angezeigt die Zahl der Blasten in Werten pro mm³ anzugeben Da jedoch nicht zu allen Zeitpunkten eine Leukozytenzählung vorlag wurde in diesen Fällen die Zahl der Blasten durch Multiplikation des relativen Blastenanteils mit dem mittels der Regressionsgeraden der Leukozyten bestimmten Wert gewonnen

5 *Aufenthaltszeit der Blasten im Blut* Da der Austritt der Blasten aus dem [illegible]

[illegible] ersten Teilung im Knochenmark vorkommt und somit der Störeffekt durch später ins Blut eintretende Zellen minimal ist Der Grenzwert für hochmarkierte Blasten wurde auf 55 Körner festgelegt, da bei höherem Wert die Zellklasse zu klein gewesen wäre um aussagekräftige Resultate zu liefern

Kasuistik

Der 23jährige Patient litt seit dem September 1966 unter einer Anämie Im September [illegible] nd eine Hepatosple[illegible] g% Ec 1,6 Mio [illegible] % als [illegible] almark war sehr zellreich und enthielt bei verdrängter Erythropoese und Megakaryozytopoese 60% Paraleukoblasten Von diesen waren 30% kleine Zellen mit schmalem Zytoplasmasaum und dichter Chromatinstruktur, 25% grosse Zellen mit promyelo-

zytoidem Aspekt und azurophilen Granula und 25% monozytoide Zellen mit breitem Zytoplasmasaum und eingebuchteten, gelappten oder nierenförmigen Kernen deren Chromatinstruktur fein granuliert oder retikulär war Die Befunde entsprachen dem, was von einigen Hämatologen als aleukämische myeloische Leukämie mit monozytärem Einschlag [9, 22], von andern als Monozytenleukämie Typ Naegeli [27] bezeichnet wird Der Patient wurde mit Bluttransfusionen, Vincristin und Corticosteroiden behandelt Die zytostatische Therapie blieb jedoch erfolglos Vincristin wurde am 24 11 1967, die Steroide am 29 11 1967 abgesetzt Am 30 11 1967 wurde zum letzten Mal Blut transfundiert Am 2 12 1967 erfolgte eine einmalige intravenöse ^{3}H Thymidin Injektion (0 1 mc/kg spez. Aktivität 2,0 mc/mM) Da die biologische Halbwertszeit von Vincristin sehr kurz ist [10] war zu diesem Zeitpunkt keine zytostatische Wirkung mehr zu erwarten Trotz des Abfalls der Blastenzahl im Blut war das Knochenmark nach wie vor sehr zellreich und enthielt zu 90% Paraleukoblasten Monozytoide Formen waren nur noch vereinzelt vorhanden Am 8 12 1967 kam der Patient zufolge profuser hämorrhagischer Diathese *ad exitum* Die Sektion bestätigte die Diagnose einer unreifzelligen Leukämie

Resultate

1. Der Markierungsindex der Interphase-Blasten im Knochenmark (markierte Blasten pro 100) ist in Abbildung 7 wiedergegeben

2 Mittlere Kornerzahl der Interphase-Blasten Die mittlere Kornerzahl aller sowie diejenige der 50% höchstmarkierten Blasten nimmt in exponentieller Weise ab Die aus den Regressionsgeraden bei semilogarithmischer Darstellung berechneten Halbwertszeiten betragen 56 respektive 51 h

3 Der Markierungsindex und die mittlere Kornerzahl der Mitosen sind in Abbildung 1 aufgezeichnet

4 Mitoseindex Auf 1000 Blasten entfallen 7,4 Mitosen Dabei wurden zur Auswertung nur Ausstriche berücksichtigt, bei denen die Kontamination des Markausstriches durch Blut gering war

5 Zahl der Blasten im Blut Wie Abbildung 2 zeigt, ist ein exponentieller Abfall der Blastenzahl mit einer Halbwertszeit von 26 h zu verzeichnen

6 Die Zahl der markierten Blasten im Blut und der Markierungsindex sind in Abbildung 3 zusammengefasst

7 Die Zahl der hochmarkierten Blasten (mit über 55 Körnern) im Blut fällt nach der 10 Stunde ab (Abb 4) Die von diesem Zeitpunkt an berechnete Halbwertszeit ist jedoch zu hoch da während der ersten Generationszeit noch hochmarkierte Zellen im Knochenmark sind welche ins Blut übertreten können Nach der 40 Stunde dagegen ist der Anteil

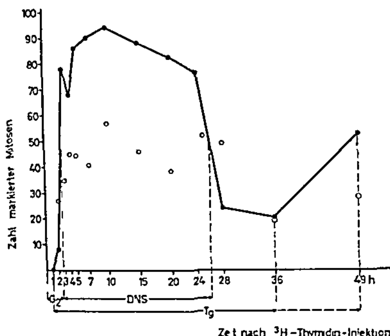


Abb 1 Markierungsindex (●) und mittlere Körnerzahl (○) der Mitosen im Knochenmark als Funktion der Zeit nach ^{3}H Thymidin Injektion. Phasen des Teilungszyklus.

dieser Zellklasse weniger als 1,5%, womit der genannte Störeffekt vernachlässigbar klein wird Die sich nun ergebende Halbwertszeit von 11 h ist daher zutreffender und wurde in den folgenden Berechnungen verwendet

Diskussion

1 Generationszeit

Aus der Kurveninterpretation lässt sich eine Generationszeit von ungefähr 40 h (40 ± 6,5) ermitteln

a) Die Markierungsindexkurve der Mitosen weist, da sie zwischen der 36 und 49 Stunde ein zweites Mal ansteigt, auf eine Generationszeit hin die zwischen 34,5 und 46,5 h liegen muss

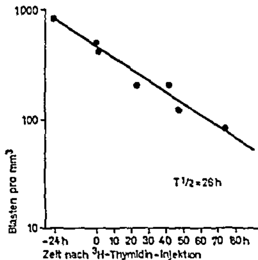


Abb 2 Zahl der Blasten im Blut unmittelbar vor und wahrend der Untersuchung

b) Die Halbwertszeit der mittleren Kornerzahl aller und die der 50% hochstmarkierten Blasten betragt 56 bzw 51 h Die Zahlen sind als obere Grenzwerte zu betrachten und stimmen daher gut mit der ersten Methode uberein

Im Vergleich dazu betragt die Generationszeit normaler Granulozytenvorlaufer nur 24 h [26] In den ubrigen bis anhin untersuchten akuten myeloischen Leukamien wurden Werte von 40 bis 84 h [6, 16, 25], in zwei Ausnahmefallen von 15 bis 30 h [21] beobachtet

2 *Phasen des Generationszyklus*

a) Pramitotische Ruhephase G2 Entsprechend der Markierungsindexkurve der Mitosen (Abb 1) betragt die mittlere Dauer von G2 2,5 h Dieser Wert stimmt mit dem anderer Autoren uberein, welche bei akuter myeloischer Leukamie ca 3 h [6, 17], bei normaler Granulopoese ca 2 h [26] fanden

b) DNS-Synthesephase Mit der oben erwahnten Methode erhalt man fur die S-Phase einen Wert von 23 h Im Vergleich zur normalen Granulopoese (14 h) [26] ist eine deutliche Verlangerung dieser Phase festzustellen Andere Autoren fanden bei myeloischer und lymphatischer Leukamie teils ahnliche (20–21,8 h) [6, 15, 17], teils kurzere Werte (4,7–20 h) [19, 21]

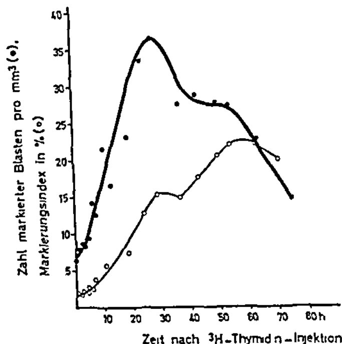


Abb 3 Zahl markierter Blasten im Blut und deren Markierungsindex als Funktion der Zeit nach ³H Thymidin Injektion

c) Mitose Anhand der Formel M/S = Mitoseindex/Markierungsindex berechnet sich eine Mitosezeit von 1,03 h Beobachtungen bei akuter myeloischer Leukämie ergaben Werte zwischen 1,1 und 1,3 h [6, 23] Demgegenüber wird für die normale Granulopoese 0,6 h angegeben [26]

d) Postmitotische Ruhephase G1 Aus dem Vorangehenden ergibt sich eine G1-Phase von ca. 14 h (normale Granulopoese ca 8 h) [26]

3 Anteil der proliferierenden und der nicht proliferierenden Zellen

Die Tatsache, dass der Markierungsindex im Knochenmark nach Ablauf einer Generationszeit höher ist als initial, weist bereits darauf hin, dass sich das Mark aus einer sich teilenden und einer sich nicht teilenden Zellpopulation zusammensetzt. Der relative Grössenanteil der sich teilenden Zellklasse kann mittels der Formel $N/N_t = t_r/S$ bestimmt

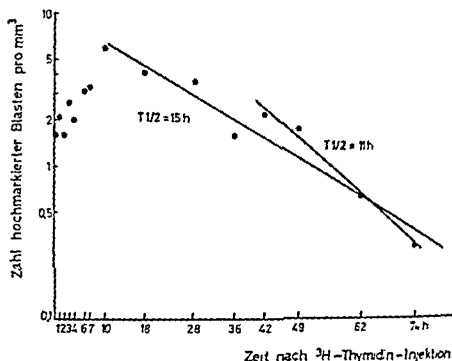


Abb 4 Zahl hochmarkierter Blasten (mit über 55 Körnern) im Blut. Halbwertszeit der hochmarkierten Blasten.

werden (N = Zahl der proliferierenden Blasten, N_s = Zahl der Blasten in der S-Phase, t_g = Generationszeit; S = DNS-Synthesezeit) Er beträgt $40 \cdot 16{,}5\%/23 = 29\%$

4 Zellproduktion im Knochenmark

16,5% der Blasten sind initial markiert, d h. sie befinden sich in der S-Phase. Die mittlere Dauer von S beträgt 23 h Es verlassen also stündlich $16{,}5\%/23 = 0{,}72\%$ Zellen diese Phase Ebenso viele Blasten treten in die Mitose ein Diese Produktionsrate von 0,72 Zellen bezieht sich auf 100 sich teilende und sich nicht teilende Blasten. Die Produktionsrate pro 100 proliferierende Blasten beträgt $^{100}/_{t_g} = 2{,}5$

Da im normalen Knochenmark 0,7 Zellen pro 100 Zellen der myeloischen Reihe und 2,4 pro 100 proliferierende Zellen gebildet werden, ist im vorliegenden Fall keine Abweichung von der Norm zu v nen In der Literatur werden für die leukämische Proliferation zwischen 0,17 und 0,59, in zwei Ausnahmefällen zwischen 0,73 bi pro 100 proliferierende und nicht proliferierende Blasten ang- [17]

Aus der Granulozytenzahl pro mm³ und der mittleren Aufenthaltszeit im Blut wurde errechnet, dass im normalen Knochenmark stündlich 5 · 10⁹ Mitosen stattfinden [?]. Unter Berücksichtigung einer Produktionsrate von 0,7% kann der Zellgehalt des normalen Markes auf ca. 7 · 10¹¹ geschätzt werden. Im vorliegenden Fall ist das Mark sehr zellreich und enthält ca. 90% Blasten. Trotz des etwas grösseren durchschnittlichen Zellvolumens beträgt die Gesamtzahl der Blasten daher ca. 10¹². Bei der oben ermittelten Produktionsrate von 0,7? % ergibt sich eine Zellproduktion von 7,? · 10⁹ Blasten pro Stunde.

5. Aufenthaltszeit der Blasten im Blut

Die Halbwertszeit hochmarkierter Blasten im Blut ($t_{1/2}$) beträgt 22 bis 15 h (mittlere Aufenthaltszeit $\bar{t}$ = 16 bis 22 h). Verglichen mit der normalen Granulozyten ($t_{1/2}$ = 6,8 h), ist in Übereinstimmung mit anderen Autoren (22 bis 25 h) [6, 16] eine deutliche Verlängerung der Aufenthaltszeit im Blut zu beobachten.

6. Ausmass des Übertritts von Blasten aus dem Mark ins Blut

Die Zahl der Blasten fällt während der Untersuchung exponentiell ab (Abb. 2). Die Konzentration (C) der Blasten im Blut kann mit folgender Gleichung beschrieben werden:

$$C_{(t)} = C_0 \cdot e^{-kt}$$

C_0 (Konzentration zum Zeitpunkt t_0) = 490 Blasten pro mm³

$k = \ln 2 / t_{1/2} = 0{,}026$

Der Austritt (E) der Blasten aus dem Blut geschieht zufällig und ist proportional der Konzentration: $E_{(t)} = \varepsilon \cdot C_{(t)}$. Entsprechend der Halbwertszeit hochmarkierter Blasten beträgt

$\varepsilon = \ln 2 / t_{1/2} = 0{,}693/$[illegible] $= 0{,}036$

Der Eintritt (I) ins Blut wird wie folgt berechnet: Die Konzentrationsänderung (dC) während des Zeitintervalls (dt) ist gleich der Differenz Influx – Efflux.

$$dC = I_{(t)} \cdot dt - \varepsilon \cdot C_{(t)} \cdot dt$$

Durch Substitution kann der Influx bestimmt werden:

[illegible]

[illegible]

... quickly ... in a de... were mounted ... stored in a covered ...

On the following day these sections were stained to demonstrate lipids and the activities of several dehydrogenases. The methods were derived with modifications from those employed by *Deane, Lobel & Romney* (1962).

Lipid preparations. The sections were fixed for ½ hour in a mixture containing 63% ethanol and 10% formalin, then stained for ½ hour in a saturated solution of Sudan black in 70% ethanol. Control sections were extracted for ½ hour in acetone at room temperature, then stained.

Aus der Granulozytenzahl pro mm³ und der mittleren Aufenthaltszeit im Blut wurde berechnet, dass im normalen Knochenmark stündlich 5 · 10⁸ Mitosen stattfinden [17]. Unter Berücksichtigung einer Produktionsrate von 0,7%/h kann der Zellgehalt des normalen Markes auf ca. 7 · 10¹¹ geschätzt werden. Im vorliegenden Fall ist das Mark sehr zellreich und enthalt zu 90% Blasten. Trotz des etwas grösseren durchschnittlichen Zellvolumens beträgt die Gesamtzahl der Blasten daher ca. 10¹². Bei der oben ermittelten Produktionsrate von 0,72%/h ergibt sich eine Zellproduktion von 7,2 · 10⁹ Blasten pro Stunde.

5 *Aufenthaltszeit der Blasten im Blut*

Die Halbwertszeit hochmarkierter Blasten im Blut ($t_{½}$) beträgt 11 bis 15 h (mittlere Aufenthaltszeit: $t_{½}/\ln 2$ = 16 bis 22 h. Verglichen mit der normalen Granulopoese ($t_{½}$ = 6,8 h), ist in Übereinstimmung mit andern Autoren (23 bis 25 h) [6, 16] eine deutliche Verlängerung der Aufenthaltszeit im Blut zu beobachten.

6 *Ausmass des Übertritts von Blasten aus dem Mark ins Blut*

Die Zahl der Blasten fällt während der Untersuchung exponentiell ab (Abb. 2). Die Konzentration (C) der Blasten im Blut kann mit folgender Gleichung beschrieben werden:

$$C_{(t)} = C_0 \cdot e^{-\gamma t}$$

C_0 (Konzentration zum Zeitpunkt t_0) = 480 Blasten pro mm³,

$\gamma = \ln 2/t_{½} = 0{,}026$

Der Austritt (E) der Blasten aus dem Blut geschieht zufällig und ist proportional der Konzentration: $E_{(t)} = \varepsilon \cdot C_t$. Entsprechend der Halbwertszeit hochmarkierter Blasten beträgt

$$\varepsilon = \ln 2/t_{½} = 0{,}693/11 = 0{,}063$$

Der Eintritt (I) ins Blut wird wie folgt berechnet: Die Konzentrationsänderung (dC) während des Zeitintervalls (dt) ist gleich der Differenz Influx – Efflux.

$$dC = I_{(t)} \cdot dt - \varepsilon \cdot C_{(t)} \cdot dt$$

Durch Substitution kann der Influx bestimmt werden:

$$I_{(t)} = C_0 \cdot (\varepsilon - \gamma) \cdot e^{-\gamma t}$$

Zu Beginn der Untersuchung betragt nach dieser Berechnung der stundliche Influx 18 Blasten pro mm^3 Unter Annahme eines Blutvolumens von 5 l ergibt sich ein Gesamteintritt von 9 10^7 Blasten pro Stunde

Da die Zellproduktion im Knochenmark (7,2 10^9 Blasten/h) 80mal grosser ist als die Zahl der Blasten, welche ins Blut eintreten (9 10^7), muss angenommen werden, dass ein bedeutender Anteil der im Knochenmark produzierten Zellen nicht ins Blut gelangt Die Beobachtung dass in den Markausstrichen gehauft pyknotische Zellen sichtbar waren, weist darauf hin, dass diese *in situ* zugrunde gehen

7 Modellvorstellungen

Im folgenden Abschnitt wird versucht, die Gesetzmassigkeiten der Zellproduktion und des Zellaustritts aus dem Knochenmark anhand einiger Modellvorstellungen zu analysieren

a) Normale Granulopoese (Abb 5, Modell N) Aus einer morphologisch nicht eindeutig abgrenzbaren Stammzellpopulation (S) treten die Zellen in ein Differenzierungs- und Teilungscompartement (Comp I) uber (Myeloblasten, Promyelozyten, Myelozyten) Nach Erreichen der Stufe der Metamyelozyten differenzieren sich die Zellen ohne weitere Mitosen aus (Comp II) Der Austritt aus dem Mark erfolgt erst am Ende des Reifungsprozesses Da Metamyelozyten, stab und segmentkernige Granulozyten kein ^{3}H-Thymidin inkorporieren, sind im Blut die ersten markierten Zellen erst 96–144 h nach der Injektion nachweisbar [16] Dieses anschaulich als «Pipeline System» bezeichnete Produktionsmuster kann die Verhaltnisse der akuten myeloischen Leukamie nicht erklaren, da hier bereits initial oder in den ersten Stunden markierte Blasten erscheinen [17]

b) Leukamische Zellproliferation *Modell 1* Austritt nach reiner Zufälligkeit der sich nicht teilenden Zellen (Abb 5, Modell 1) Fur die akute myeloische Leukamie schlug KILLMANN folgendes Modell vor [17] Analog zur normalen Granulopoese enthält das Knochenmark eine Population sich teilender und eine sich nicht teilender Blasten Der Übertritt ins Blut wird ebenfalls fast ausschliesslich von sich nicht teilenden Zellen vollzogen Dies wird angenommen, weil bei allen Leukamien der initiale Markierungsindex im Blut niederer ist als im Knochenmark Im Unterschied zur normalen Hämatopoese erfolgt der Zellaustritt aus dem Comp II jedoch nach reiner Zufalligkeit

Die mathematische Formulierung dieser Modellvorstellung führt im vorliegenden Fall zu folgenden Gleichungen

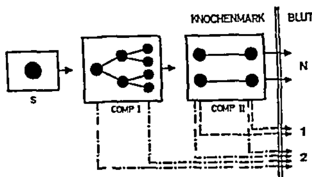


Abb 5 Schema der Zellproduktion und des Zellaustritts der normalen Granulopoese (N) und der Modelle 1 und 2.

α) Markierungsindex (MI) im Comp II

Bis zur 3 Stunde enthält das Comp II keine markierten Blasten. Diese durchlaufen G2 und M des Comp I

Von der 3 bis zur 26. Stunde treten stündlich 0,72/71 = 1,01% markierte Zellen ins Comp II (71% aller Blasten) Der Austritt aus diesem Pool ist proportional der Konzentration Die Änderung des Markierungsindex (dMI) beträgt daher

$$dMI = 0{,}0101 \cdot dt - 0{,}0101 \cdot MI_{(t)} \cdot dt.$$

Diese inhomogene Differentialgleichung wird nach LAGRANGE gelöst.

$$MI_{(t)} = 1 - e^{-0{,}0101(t-3)}.$$

Von der 26. bis zur 42. Stunde treten keine markierten Blasten hinzu. der MI fällt exponentiell ab

$$MI_{(t)} = MI_{26} \cdot e^{-0{,}0101\,(t-26)}$$

Darnach gelten bis zur 65 Stunde wiederum die Bedingungen der ersten, in der Folge bis zur 84 Stunde diejenigen der zweiten Gleichung

Nachdem der Anstieg des MI im Comp II durch diese Gleichungen festgelegt ist, kann die zu erwartende Zahl markierter Blasten im Blut berechnet werden.

β) Der Influx aller Blasten ins Blut beträgt

$$I_{(t)} = C_0(\varepsilon - \gamma)e^{-\gamma t}$$

γ) Der Influx markierter Blasten (I*) ist daher

$$I^*_{(t)} = MI_{(t)} \cdot C_0(\varepsilon - \gamma)e^{-\gamma t}$$

Der Austritt aus dem Blut erfolgt zufällig und ist somit proportional der Zahl markierter Blasten im Blut. Daraus ergibt sich für die Konzentrationsänderung markierter Blasten (dC*)

$$dC^{*} = C_0 \cdot MI_{(t)} (\varepsilon - \gamma) e^{-\gamma t} dt - \varepsilon C^{*}_{(t)} \cdot dt.$$

Diese Gleichung wurde mit dem Digitalcomputer gelöst, wobei sich das Mimic-stem [1] als einfachste, auch für einen Mediziner zu bewältigende Computersprache erwies.

Modell 2 Austritt nach reiner Zufälligkeit aller Blasten (Abb 5, Modell 2) Dem zweiten Modell wird die Vorstellung zugrunde gelegt, dass sowohl die Zellen des Comp I, als auch die des Comp II zufällig austreten, die Zellproduktion im übrigen aber dem Modell 1 entspricht Analog zum Modell 1 lässt sich das Produktionsmuster durch ein Gleichungssystem beschreiben und mit dem Computer lösen

Modell 3 Ein weiteres Modell wird ausgehend von folgenden Beobachtungen konzipiert

α) GAVOSTO [12] unterteilte die Zellen des Knochenmarks bei akuter myeloischer Leukämie entsprechend ihrem Zelldurchmesser in 3 Grossenklassen Dabei betrug der initiale MI der grossen Zellen 50%, der mittleren 5% und der kleinen 0% Nach einer Generationszeit sank der MI der grossen Blasten auf ca 10% ab Dies weist darauf hin, dass unmarkierte Blasten zu dieser Zellklasse hinzutreten (Transformation von mittleren oder kleinen in grosse Blasten) GAVOSTO folgert daraus mit Recht, dass diese grossen Blasten nicht Stammzellen sein können Die Frage bleibt offen, ob es sich bei den transformierten Zellen um Stamm-, G1- oder G0 Zellen handelt Der Abfall des MI in dieser Untersuchungsserie ist so ausgeprägt, dass er schwerlich durch das Hinzutreten von G1-Zellen bei rascher Asynchronisation erklärt werden kann Bedenkt man anderseits, dass im Proliferationscompartement aus einer Stammzelle durch n sukzessive Teilungen 2^n Zellen entstehen, ist das Ausmass des möglichen Stammzellnachschubes offensichtlich zu klein, um den raschen Abfall der initial stark markierten Zellpopulation zu erklären Ein Übertritt aus einem G0-Pool ist demgegenüber viel wahrscheinlicher

β) Die Kurve des Markierungsindex der Mitosen zeigt bei allen bisher untersuchten Leukämien wohl ein erstes, meist aber kein zweites klar abgrenzbares Plateau Dieses Verhalten kann ebenfalls durch das Eintreten von initial sich nicht teilenden und somit nicht markierten Zellen in den Teilungszyklus erklärt werden Aufgrund dieser Beobachtungen sowie der Erfahrungen mit andern Zellsystemen [11] muss angenommen werden, dass nicht proliferierende Zellen auf entsprechende Stimuli hin erneut in den Teilungszyklus eintreten können

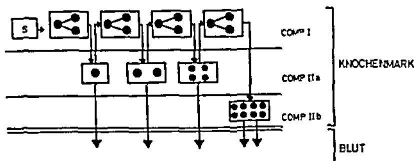


Abb 6 Schema der Zellproduktion und des Zellaustritts entsprechend Modell 3

Im Modell 3 (Abb 6) werden wiederum sich teilende (Comp I) und sich nicht teilende Zellen (IIa, IIb) unterschieden. Analog zur normalen Granulopoese durchlaufen die Zellen sukzessive Teilungszyklen, um so zu Endzellen heranzureifen Nach jeder Mitose jedoch treten die Zellen in eine Ruhephase G0 (Comp IIa) ein und verweilen dort, bis sie auf entsprechende Stimuli hin wiederum den nächsten Teilungszyklus beginnen Die Transformation von G0-Zellen in G1-Zellen ist ein Zufallsprozess Zellen beider Untergruppen des Comp II treten zufällig ins Blut über

Die Grösse des Comp IIa wird im Modell so gewählt, dass sein Markierungsindex nach 49 tg Stunden auf 58% ansteigt Damit erreicht der MI der Mitosen, welche sich aus diesem Comp rekrutieren, nach 49 h wiederum diesen Wert, wie es in der Untersuchung der Fall ist. Aus dem Konzept ergibt sich, dass durchschnittlich pro Zellteilung eine G0- und eine Endzelle entsteht Der Verlust von G0-Zellen durch Übertritt ins Blut beeinflusst dieses Verhältnis nur wenig und wird daher vernachlässigt.

Die mathematische Formulierung des Modells führt zu folgenden Gleichungen.

Der MI der beiden Comp IIa und IIb wird durch den Zufluss markierter Blasten aus dem Comp I und dem jeweiligen Austritt aus den Compartementen bestimmt. Die Zellproduktion beträgt 0,72 %/h, der relative Zelldurchfluss durch Comp IIa 0,72/17 = 0,042, durch das Comp IIb 0,72/64 = 0,0113

a) der MI im Comp IIa nimmt folgende Werte an

t 0–3 h $MI = 0$

t 3–26 h $dMI = 0{,}042\ dt - 0{,}042\ MI\ dt,$
$MI = 1 - e^{-0{,}042(t-3)}$

t 26–29 h $dMI = -0{,}042\ MI\ dt$
$MI = MI_{26}\ e^{-0{,}042(t-26)}$

t 29–52 h, $dMI = 0{,}042\,(1-e^{-0{,}042(t-29)})\,dt - 0{,}042\ MI\ dt$,
t 52–55 h, $dMI = 0{,}042\ MI_{26}\ e^{-0{,}042(t-52)}\,dt - 0{,}042\ MI\ dt$
t 55–78 h, $dMI = 0{,}042\ MI_{(t-26)}\,dt - 0{,}042\ MI\ dt$

b) Der MI im Comp IIb kann auf folgende Weise definiert werden

t 0– 3 h, $MI = 0$,
t 3–26 h, $MI = 1-e^{-0{,}0113(t-3)}$,
t 26–29 h, $MI = MI_{26}\ e^{-0{,}0113(t-26)}$,
t 29–52 h, $dMI = 0{,}0113\,(1-e^{-0{,}042(t-29)})\,dt - 0{,}0113\ MI\ dt$,
t 52–55 h $dMI = 0{,}0113\ MI^{*}_{(26)}\ e^{-0{,}042(t-52)}\,dt - 0{,}0113\ MI\ dt$,
t 55–78 h, $dMI = 0{,}0113\ MI^{*}_{(t-26)}\,dt - 0{,}0113\ MI\ dt$
(MI^{*} = MI des Comp IIa)

Da so der MI in den beiden Untergruppen des Comp II festgelegt ist kann in gleicher Weise wie beim Modell 1 das Erscheinen markierter Blasten im Blut berechnet werden

Anmerkung Alle Modelle basieren auf der Annahme dass die Zahl der Blasten im Knochenmark konstant und der Zelltod im Mark ein Zufallsprozess ist Die Vernachlassigung des altersabhängigen Zellunterganges bewirkt in den Modellen einen zu langsamen Anstieg der Markierungsindexkurve Der Vergleich der Modelle mit dem Experiment zeigt jedoch, dass dieser Fehler nicht sehr bedeutend sein kann

Die extramedullare Granulopoese wird in den Modellen nicht gesondert berücksichtigt. Ein grober Fehler ist dadurch nicht zu erwarten da erstens nur eine geringe Hepatosplenomegalie besteht und zweitens die leukamischen Blasten in Leber und Milz kaum prinzipiell andern Gesetzen gehorchen

8 *Vergleich der Modelle mit dem Experiment*

a) Markierungsindex im Knochenmark

In Abbildung 7 wird der Markierungsindex der Blasten des Knochenmarkes, wie er sich aus den Modellen 1, 2 und 3 ergibt, den experimentell gefundenen Werten gegenubergestellt Samtliche Modelle zeigen einen etwas starkeren Anstieg Dies mag dadurch bedingt sein, dass die Untersuchungsmethode diejenigen Zellen nicht erfasst, die nach einer Teilung unter den Markierungsgrenzwert fallen, und somit zu niedere experimentelle Werte liefert

Obwohl Modell 3 mit dem Experiment am besten übereinstimmt, kann es aufgrund dieses Kurvenvergleichs noch nicht bevorzugt werden Im Verhaltnis zu den methodischen Fehlern weichen die Modelle zu gering voneinander ab

b) Markierte Blasten im Blut

In Abbildung 8 wird die Anzahl der markierten Blasten im Blut, die sich aus den Modellen ergibt, mit den experimentellen Resultaten vergli-

einfachen und dem des doppelten Chromosomensatzes liegt. Die Zahl der Zellen, die im Blut die weitere DNS-Synthese einstellen, kann jedoch in ihren Versuchen auf höchstens 50% geschätzt werden.

Die beste Übereinstimmung mit den beobachteten Werten bringen die Modelle 3 und 3a. Modell 3a ist eine Modifikation von Modell 3, bei der angenommen wird, dass 5% statt nur 3% der im Blut erscheinenden Blasten aus dem Comp. I entstammen. Dies erscheint gerechtfertigt, da nur so der Anstieg während den ersten 3 Stunden erklärt werden kann. Entsprechend den vorausgehenden Ausführungen musste für diese Zellgruppe ein partieller Verlust (<50%) der DNS-Synthesefähigkeit nach Eintritt ins Blut postuliert werden.

Modell 3 gewinnt um so mehr an Wahrscheinlichkeit, als es 1. die Zahl markierter Blasten im Blut, 2. den Markierungsindex des Knochenmarkes am zutreffendsten beschreibt, 3. eine Erklärung gibt für den auch von andern Autoren festgestellten Verlauf der Markierungsindexkurve der Mitosen und 4. den raschen Abfall des MI der grossen, initial markierten Blasten im Knochenmark erklärt [12].

Schlussfolgerungen

Die zytokinetischen Befunde sprechen im vorliegenden Fall für folgende Knochenmarkorganisation: 1. Das Knochenmark enthält proliferierende und nicht proliferierende Zellen. 2. Es bestehen Hinweise dafür, dass die proliferierenden Zellen nach jeder Mitose vorübergehend in eine Ruhephase G0 übertreten und in dieser unterschiedlich lange verweilen, bis sie den nächsten Teilungszyklus beginnen. 3. Die prozentuale Zellproduktion ist gleich gross wie diejenige der normalen Granulozytenvorläufer. 4. Die Generationszeit sowie die Phasen des Teilungszyklus sind verlängert. 5. Die mittlere Aufenthaltszeit der Blasten im Blut ist verlängert. 6. Ein bedeutender Anteil der im Knochenmark gebildeten Zellen tritt nicht ins Blut über. Dies wird als Ursache der aleukämischen Verlaufsform betrachtet. 7. Der Übertritt ins Blut kann nicht nur von Endzellen sondern auch von G0-Zellen vollzogen werden. Vereinzelt treten wahrscheinlich auch proliferierende Zellen ins Blut über.

Im normalen Knochenmark ist der Austritt der Zellen vom Differenzierungsgrad abhängig und als aktive Diapedese zu verstehen [20]. In der hier untersuchten Leukämie treten erstens nicht alle produzierten Blasten aus und ist zweitens der Austritt nicht auf Endzellen beschränkt.

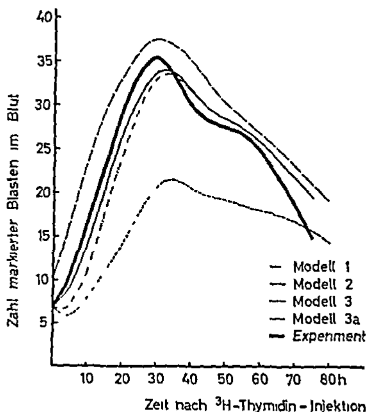


Abb 8 Zahl der markierten Blasten im Blut (pro mm³) Vergleich der Modelle mit dem Experiment

zu hohe Werte, im Experiment steigt die Zahl der markierten Blasten erst nach der 4 Stunde rasch an Dies spricht gegen die im Modell angenommene betrachtliche Ausschwemmung aus dem Comp I Zudem stimmt ein weiteres Postulat dieses Modells mit der Realitat nicht uberein Wenn namlich auch proliferierende Zellen nach reiner Zufalligkeit aus dem Knochenmark austreten wurden, so mussten erwartungsgemass der initiale MI im Knochenmark und im Blut gleich gross sein Dies ist jedoch nicht der Fall Dieser Unterschied zwischen den MIs des Knochenmarks und des Blutes kann schwerlich durch die zusatzliche Annahme erklart werden, dass die proliferierenden Blasten nach Eintritt ins Blut die DNS-Synthesefahigkeit verlieren HALE und COOPER [14] weisen zwar auf solche Vorkommnisse hin Sie haben im Blut bei akuten Leukamien Zellen gefunden, die einerseits kein ^{3}H-Thymidin inkorporieren, andererseits aber einen DNS Gehalt aufweisen, der zwischen demjenigen des

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Dieses veränderte Verhalten kann als Differenzierungsdefekt der leukämischen Blasten verstanden werden

Die Tatsache, dass bei der normalen Granulopoese das zweite P teau der Markierungsindexkurve der Mitosen wiederum annähernd a 100% ansteigt [26], spricht dafür, dass hier eine allfällige G0 Popu tion wesentlich kleiner sein musste als bei den untersuchten Leukämie Unter der Annahme, dass die Zellproduktion durch die Grosse der G Population reguliert wird [18], kann die Vermehrung der G0-Zellen a Ursache der Inhibition der normalen Granulopoese betrachtet werde Dass die relative Grosse der G1- (oder G0-) Population bei Änderung d Zellproduktion tatsachlich variiert, geht aus den Untersuchungen v HANNA *et al* [13] hervor, welche bei der Ratte bei gesteigerter Er thropoese eine Verkurzung der Generationszeit, insbesondere d G1-Phase der Erythroblasten fanden

Zusammenfassung

Nach einmaliger *in vivo*-³H Thymidin Markierung wird die Zytokinetik eir aleukamischen akuten myeloischen Leukämie bestimmt Verglichen mit der norm len Granulopoese sind die Generationszeit, die einzelnen Phasen des Teilungsz klus und die Aufenthaltszeit der Blasten im Blut verlangert, während die relati Zellproduktion gleich gross ist Von den im Knochenmark produzierten Blast tritt nur ca. 1/44 ins Blut über Dies wird als Ursache der aleukämischen Verlau form betrachtet Ein Vergleich der Resultate des Knochenmarkes und des Blut mit mathematischen Modellen führt zu einem hypothetischen Produktionsmode bei dem die proliferierenden Blasten nach jeder Mitose eine G0-Phase durchlauf und End und G0-Zellen ins Blut übertreten

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A Study of ^{51}Cr-Labelled Platelets and the Chromosomal Pattern in a Case of Primary Haemorrhagic Thrombocythaemia

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Abstract. In a patient with primary haemorrhagic thrombocythaemia the initial disappearance of compatible donor platelets from the peripheral blood was markedly increased compared to that of the autologous platelets. Both autologous and heterologous platelets disclosed abnormally short life span. The initial platelet disappearance rate may be used as a supplementary examination for characterisation of platelet abnormalities. In the peripheral blood an unexpected number of cells with structural chromosome aberrations were found. These aberrations were supposedly induced by busulphan therapy 6 years earlier. No clone formation or Ph¹-chromosomes were found in the bone marrow.

Key Words
Haemorrhagic thrombocythaemia
Karyotype
Platelet life span

Primary haemorrhagic thrombocythaemia is a rare myeloproliferative disorder characterized by a definite clinical picture [9, 19, 21]. This consists of permanently elevated platelet counts, megakaryocytic hyperplasia of the bone marrow, recurrent spontaneous haemorrhages and often vascular thrombosis and splenomegaly. Frequent concurrent findings are a slight neutrophilic leucocytosis with a 'shift to the left', iron deficiency anaemia during the haemorrhagic phases of the disease alternating with a slight erythrocytosis, a moderate eosinophilia and/or basophilia, platelet anisocytosis and the occurrence of agglutinated platelet masses in slide preparations. Also the myelocytic and erythrocytic elements in the bone marrow may be hyperplastic. Hepatomegaly is often seen. The mucous membranes of the gastrointestinal tract, the upper respiratory tract and the urinary tract are the most common sites of bleeding. Bleedings

due to slight trauma or spontaneously into the s c tissues are very common The spontaneous bleedings may be preceeded by small thrombosis occuring mostly in the veins, expecially in the splenic vein, eventually resulting in infarction or atrophy of the spleen

The purpose of the present study was to investigate platelet abnormalities using the ^{51}Cr labelling technique and further to examine blood and bone marrow for chromosome aberrations These investigations were performed during a subtotal *clinical* remission of the disease, 6 years after treatment with busulphan

Case History

The patient was a 75 year old woman, who presented herself in 1949 with melaena and a history of recurrent venous thromboses of the legs Between 1949 and 1960 she had 12 episodes of gastrointestinal bleedings which on 2 occasions required blood transfusions Numerous radiological examinations of the gastrointestinal tract were done in this period, but only once a definite prepyloric ulcer was demonstrated All the time she suffered from cutaneous and subcutaneous haemorrhages and since 1954 a moderate splenomegaly was obvious

Ever since 1949 iron deficiency anaemia alternating with slight erythrocytosis was present White cell counts were between 14 000 and 30 000/μl and differential counts showed leftshifted neutrophilia Platelet counts between 1 2 and $8\,2\times10^6/\mu l$ were noted during the years *1957–1960* Several bone marrow specimens showed moderate hyperplasia of red and white cell precursors and a greatly increased number of megakaryocytes with nuclear hypersegmentation and eventually pyknosis Coagulation factors, coagulation time and bleeding time (DUKE) were found to be normal

Based on these clinical and laboratory findings and the abscence of leukaemic or myelofibrotic changes in the peripheral blood and the bone marrow the diagnosis of *primary haemorrhagic thrombocythaemia seemed secured* Busulphan treatment was consequently started in October 1960 resulting in normalization of platelet, red and white cell levels within 7 weeks During this treatment the previously positive reactions for blood in the stools became constantly negative the s c bleedings decreased and the spleen diminished in size This treatment was discontinued in January 1962

One year later a Billroth I resection of the stomach was performed because of the repeated finding of a prepyloric ulcer but although macroscopic gastrointestinal bleedings did not occur, intermittent iron deficiency anaemia was still present

In September 1968 she was readmitted to hospital because of colonic dyspepsia Clinical examinations revealed splenomegaly and s c bleedings but tests for blood in the stools were negative A moderate normocytic hypochromic anaemia was present platelet counts were about $0\,4\times10^6/\mu l$ and white cells were about

30 000/µl. Slide preparations revealed agglutinates of platelets and moderately leftshifted neutrophilia. Bone marrow examinations in October gave no conclusive diagnosis. Chromosome analysis was performed as described below, succeeded by the platelet labelling studies.

The patient died suddenly in March 1969, due to pneumonia and heart failure before planned repeated platelet studies could be carried out.

Autopsy showed diffuse bronchopneumonia and pulmonary oedema. The spleen was enlarged measuring 20 × 15 × 14 cm, weight 1,300 g, uniformly red without focal changes. The liver was moderately enlarged, without macroscopic signs of cirrhosis or metastases. The kidneys were granulated with a few small white infiltrations and a few larger cysts containing fluid resembling urine. The bone marrow was reddish and hyperplastic. All other findings were due to usual senescence.

Microscopy of the liver and spleen showed widespread extramedullary haematopoiesis with many megakaryocytes. Sections of the lungs showed stasis, oedema and pneumonia and in many capillaries megakaryocytes. Sections of the kidneys showed glomeruli with focal hypercellularity, fibrinoid necrosis and in many areas destruction of blood vessels, infiltration with polymorphs and in many places megakaryocytes. No signs of haematopoiesis in the interstitial tissue or of thrombotic microangiopathia. The findings were consistent with focal embolic glomerulonephritis. Sections of bone marrow showed maximal hyperplasia of the marrow elements and presence of numerous megakaryocytes of varying size and polymorph appearance. The cytoplasm was weakly eosinophilic, containing very small granular elements. There were very slim osseus trabeculae and no signs of bone marrow fibrosis (a more detailed description of the pathological findings at the autopsy of this patient has recently been published elsewhere [11]).

Special Investigations

The rationale for undertaking special studies of the platelets of this particular patient would be, that only few details are known about platelet life span in this rare disease, and the results have been very contradictory [2, 7, 9, 13, 18]. In addition to this the initial uptake of platelets in the reticulo-endothelial system of a patient like this has to our knowledge not been investigated. This uptake, which primarily takes place in the spleen, is reflected in the peripheral blood circulation as the initial recovery phase, which we preferred to measure in our patient.

Initial recovery and turnover of ^{51}Cr labelled platelets. Platelets were labelled with ^{51}Cr-chromate using Abrahamsen's [1] modification of an earlier method. 2 investigations were carried out on the patient, one using her own platelets and one using platelets from a normal donor. The interval between the 2 investigations was 1 month. For comparison, platelets from a normal donor were given to a control patient without haematological or vascular disease. In all instances the initial disappearence of the platelets from the peripheral blood an the platelet turnover were followed after the termination of a very rapid i.v. injection of labelled platelets. All peripheral blood samples were taken through a polyethylene catheter in the femoral artery.

As shown by other investigators, the maximum recovery of platelets occurs approximately one minute after the termination of the platelet injection [7]. In the study of the initial recovery phase, the blood counting rate 1 min after the termi

nation of the injection was chosen to indicate 100% radioactivity The comparability of this initial maximum value in the 3 investigations was checked by calculating the dilution which had occurred at this time and only minor differencies were found (2% difference between the 2 initial values in the investigations in the patient) The blood volume was not measured

In the study of platelet turnover, the blood counting rate one day after the injection was chosen to indicate 100%

All radioactive counting rates were corrected for background and red cell radioactivity, altogether about 10% of the maximum counting rate

Chromosome studies Chromosome investigations on patients with primary thrombocythaemia have only been reported in very few cases and in most of these no chromosome abnormalities have been found The aim of the present chromosome study was to investigate (a) if clone formations with a specific chromosomal pattern could be found in the bone marrow, (b) if any signs of development into leukaemia could be found, (c) if treatment with busulphan had caused chromosome abnormalities

The specimens of bone marrow were examined by a direct method without culture of the cells The blood samples were treated in the customary way, with a culture period of 48 h [16]

Results

Initial recovery of platelets from the blood The most marked finding was the quicker and more complete disappearance of platelets from the peripheral blood of the patient as compared to the blood of the normal control This uptake was more pronounced when the patient received normal compatible donor platelets than when her own platelets were given (fig 1) Our initial disappearance values, in the normal person, of approximately 50% after 15–20 min correspond to the values obtained by ASTER [3]

Platelet turnover As it appears from figure 2 the platelet turnover curve in the control case had a characteristic, linear configuration indicating that the platelets had a definite life span, which might be calculated – in this case about 8 days – corresponding to a breakdown of approximately 40,000 platelets/μl/day This is in accordance with previous data in normal subjects [1, 7, 13, 18]

The turnover curve in the patient showed a bent, abnormal configuration, and a more rapid turnover (fig 2) This was especially pronounced when she received her own platelets This points towards an abnormally fast, random removal of both normal and 'thrombocythaemic' platelets, the latter being most pronounced

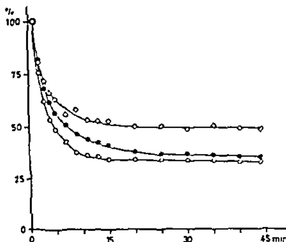


Fig 1 Initial recovery of ^{51}Cr labelled platelets from the peripheral blood. ○ Donor platelets in normal control ◌ Donor platelets in patient ● Patient platelets in patient

Chromosome analysis Thirteen cells from the bone marrow were analysed These showed a normal female karyotype, and contained no numerical or structural chromosome aberrations, particularly no cells with Philadelphia chromosomes

From the peripheral blood, 50 cells were analysed No numerical aberrations were found However, cells with characteristic, structural chromosome aberrations were found 5 cells (10%) contained dicentric chromosomes and acentric fragments (fig 3) 2 cells contained stable chromosome aberrations with translocations

Discussion

Radioactive platelets studies In this case of primary haemorrhagic thrombocythaemia it was our intention to investigate whether the spleen, or the reticulo-endothelial system seemed to retain platelets in an abnormally high degree during the first minutes after platelet injection, as it has been shown in other diseases with splenomegaly [3] In addition, we wanted to know whether platelets from a normal donor were treated dif-

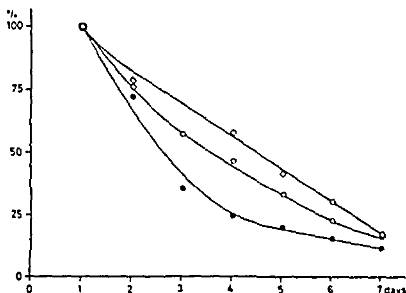


Fig. 2. Turnover of ^{51}Cr labelled platelets. △ Donor platelets in normal control. ○ Donor platelets in patient. ● Patient platelets in patient.

ferently in the patient's organism than her own platelets during the first minutes. If so, this could support theories about a possible abnormality of the platelet surface [6, 14, 15]. It seems as if this decreased adhesiveness may be one of the very few measurable differences between this rare thrombocytopathy, other platelet abnormalities and thrombocytosis. No specific surface abnormalities could be detected by ultrastructural studies, which only revealed changes in the internal organs of the platelets common to various kinds of thrombocytopathies and disturbed thrombocytogenesis [4, 12], the meaning of which is still unknown.

The results demonstrate an increased initial disappearance from the peripheral blood of both donor and patient platelets compared to the disappearance in a control person (fig. 1). However, there was a marked difference in the initial disappearance rate of the 2 kinds of platelets from the peripheral blood (50% of the donor platelets disappeared from the peripheral circulation in approximately 4 min and 40 sec, while the same disappearance value using patient platelets was not reached until approximately 7 min and 20 sec).

One reason for this difference might be platelet isoimmunisation due to earlier blood and platelet transfusions. This seems rather unlikely as isoimmunisation usually causes a very marked reduction of platelet life

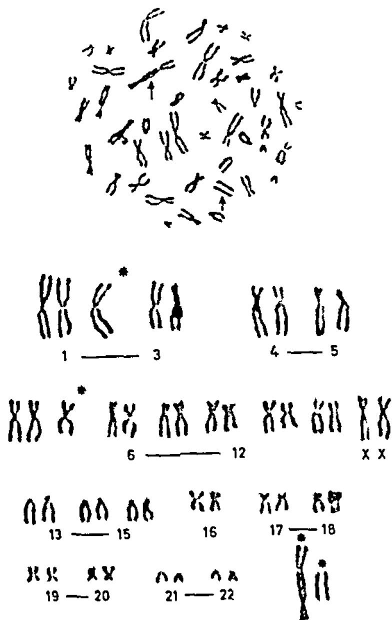


Fig. 3. Cell from peripheral blood from patient treated with busulphan 6 years previously. Karyotype showing dicentric chromosome and acentric fragment.

span [5], which we did not find Another explanation might be that the donor platelets had been damaged to a higher degree than the patient platelets during the labelling procedure This factor cannot be ignored, but does not seem likely, because the proportion between the initial and the first day counting rates was the same in the 2 studies Different adrenalin levels in the patient's blood during the platelet injections might also account for differences in the recovery of platelets in the peripheral blood [3] However, the 2 studies were undertaken at the same time of the day under precisely similar conditions using lidocain 1% without adrenalin for local anaesthesia before introducing the polyethylene catheter in the femoral artery In our opinion, the differences between the initial disappearance rates might point towards a subnormal platelet surface adhesiveness of the 'thrombocythaemic' platelets

It is proposed that the described technique using radiochromium labelled platelets to measure the disappearance rate from peripheral blood circulation during the first 15–20 min after platelet injection, might be used as a supplement to the existing *in vivo* techniques for measuring platelet adhesiveness in cases with suspected platelet surface abnormalities

The platelet survival studies confirmed the findings of LANDER and DAVEY [13] in one patient and of DAVEY [7] in 2 out of 6 patients with thrombocythaemia exhibiting shortened platelet life span Other studies did not show this [2, 18] In our patient, an abnormally short life span of normal platelets was also demonstrated This finding seems to be a rather unspecific phenomenon as it has been found in a great many platelet and splenic diseases [1, 5, 17], in venous and arterial occlusive states uraemia, hepatic cirrhosis and other diseases The observed differences of survival time in the present study between patient and donor platelets in the patient's organism do in fact reveal an abnormality of her platelets

Chromosome studies Several authors have carried out chromosome investigations on patients with various disorders within the myeloproliferative syndrome, including also patients with primary thrombocythaemia In most cases, no abnormalities have been found, but specific clone formations in the bone marrow have been reported in rare cases Thus, in an untreated patient with thrombocythaemia ROWLEY and BLAISDELL [20] detected a clone formation with 48 chromosomes, amounting to 11% of the analysed cells in the bone marrow However, it should be mentioned that clone formations in patients with myeloproliferative dis-

cases generally amount to more than 50% of the analysed cells in the bone marrow [22] As stated above, no clone formation with abnormal chromosome pattern was found in the bone marrow from our patient

Galton [8] and Hellriegel [10] *inter alia* reported cases interpreted as primary thrombocythaemia in which Philadelphia chromosomes were later on detected in bone marrow cells It is extremely difficult to assess whether we are here dealing with cases of primary thrombocythaemia developing into chronic myeloid leukaemia or with chronic myeloid leukaemia which initially presented signs of primary thrombocythaemia Anyhow these problems clearly indicate the necessity of carrying out chromosome analyses on all patients with thrombocythaemia Likewise the close relationship between primary thrombocythaemia and polycythaemia is shown by the not uncommon development of polycythaemia following the treatment of an initially severely anaemic patient. These cases can also develop into leukaemia probably acute leukaemia in which bone marrow cells may contain clones with abnormal chromosome pattern [22] As stated above no Philadelphia chromosomes or other chromosome abnormalities were found in our patient which might arouse suspicion of leukaemia

The types of structural chomosome aberrations found in the peripheral blood of our patient are well known First and foremost they have been studied in connection with radiation exposure but it is known that they may be induced by a number of cytostatic agents Ostensibly our patient had received no kind of radiation treatment (particularly not ^{32}P) and consequently the chromosome aberrations are supposed to be induced by the busulphan treatment However this treatment was terminated 6 years before the chromosome analyses were carried out and therefore the number of aberrations fourd is *remarkably high* It is known from experiments with radiation induced chromosome aberrations that the number of cells with unstable aberrations *declines rapidly* with time after exposure and the relatively high proportion of cells with aberrations found in our patient was not to be expected 6 years after treatment However no certain significance can be ascribed to these figures since the number of investigated cells were rather low

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Acta haemat 46 188-192 (1971)

New Erythrocyte Glucose-6-Phosphate Dehydrogenase Variant

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Diseases (Head Prof J CZYZEWSKA) School of Medicine, Wroclaw

Abstract Glucose 6 phosphate dehydrogenase from a patient who had a hemolytic episode during infection was purified and characterized The enzyme differs by its properties (activity electrophoretic mobility affinity for glucose 6-phosphate thermostability and pH optimum) from the normal glucose 6-phosphate dehydrogenase type B and from the other known variants

Key Words
Glucose 6-phosphate dehydrogenase
Erythrocyte metabolism
Hemolytic anemia

Over the last years, defects of human erythrocyte glucose 6-phosphate dehydrogenase (G6PD) have attracted attention both from a clinical and biochemical point of view The properties of 2 normal phenotypes of G6PD Gd(+)B, common in various populations and Gd (+) A in Negroes have been described About 15 variants of G6PD different from A and B have been characterized in patients with congenital nonspherocytic hemolytic disease most of them among Negro, Mediterranean and Middle East populations [3, 10] In Poland, only sporadic cases of G6PD deficiency were reported and none of them was characterized until now In this communication the properties of one of the variants are presented

Materials and Methods

The patient is a boy 6 years old During 3 weeks after birth severe neonatal jaundice was evident but no laboratory and enzyme studies were performed Up to 5 years no clinical manifestations were observed Recently an acute hemolytic crisis occurred as a response to infection of the upper respiratory tract It was accompanied by severe jaundice progressive hemolytic anemia and hemoglobinuria

The family of the patient lives in the country near Wroclaw, in the western part of Poland. His parents are born in the south-eastern part of Poland (the district of Lvov). They have no relatives among people of mediterranean origin.

The activity of G6PD was determined spectrophotometrically [8]. The enzyme was partially purified by the method of KIRKMAN [4] and characterized as recommended by the WHO Standardization Committee [10].

The NADPH generating capacity of the erythrocytes was studied in the mother of the patient by the ascorbate cyanide test and by measuring the glutathione reductase activity in red cells after incubation with chromate [11].

Electrophoresis was carried out on starch gel using 75 mM tris citrate buffer pH 8.3 for gel and 230 mM borate and 35 mM NaOH buffer pH 8.3 for reservoirs. Staining: filter paper saturated with a mixture containing 2 mg of glucose-6-phosphate (G6P), 5 mg of nicotine adenine dinucleotide phosphate (NADP), 5 mg of nitrotetrazolium blue, 0.1 mg of phenosine metasulphate and 0.1 mm of $MgSO_4$ in 10 ml of 50 mM triethanolamine HCl buffer pH 7.5 was overlayed on the sliced gel.

For optimum pH determination 50 mM veronal acetate buffers ranging from pH 5.0 to 6.0, triethanolamine HCl from pH 7.0 to 9.5 and veronal-carbonate HCl for higher pH values were used.

Thermostability determination of G6PD was performed at 45 °C. Phosphohexoseisomerase activity was measured according to BODANSKI [9], phosphofructokinase and aldolase activities by the modified method of BRUNS [1].

Results

The activity of glucose-6-phosphate dehydrogenase was measured in hemolysate several times during a 6-month period after the hemolytic episode. It was 0.3–0.4 IU/g Hb which is equal to 7–10% of normal values. According to the WHO Standardization Committee this type of deficiency should be indicated by the symbol Gd (–).

For further characterization of the G6PD variant the enzyme was purified and its properties were studied. The results are summarized in figure 1. K_M value for G6P (110 μm) is higher than that observed in normal erythrocyte glucose-6-phosphate dehydrogenase (50–78 μm). K_M for NADP (5.6 μm) does not differ from normal values (1.7–15 μm) [3, 10]. The enzyme has a low electrophoretic mobility, about 75% of that of normal type B. The pH curve differs slightly from the normal one but is not biphasic as it has been observed in most of the known variants. The thermal stability of the enzyme is markedly diminished. The G6PD loses all its activity after 20 min incubation at 45 °C and 80% of the initial activity after 3 h standing at room temperature.

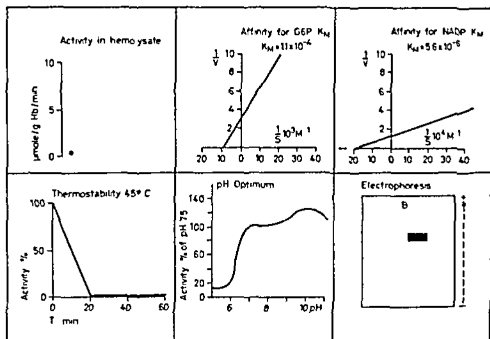


Fig. 1 Characteristics of G6PD of the patient The shadowed area indicates the normal range

Discussion

It seems that in this case the decreased G6PD activity is not due to the diminished rate of the synthesis of the normal enzyme According to PINTO *et al* [6] this variant cannot be included in the group of the deficiencies of the I type The variant observed in our patient differs from the normal type B by its properties and presumably by its structure The difference in electrophoresis seems to be due to the change in the amino acid composition of the enzyme molecule The affinity for coenzyme is normal, but that for substrate is slightly diminished The enzyme is very unstable and its low activity in erythrocytes may be attributed to its thermolability

G6PD of the patient differs from all the previously described variants The common deficient enzyme variant Gd (–) Mediterranean has the electrophoretic pattern identical to the type B increased affinity for G6PD and biphasic pH curve The low electrophoretic mobility accompanied by marked thermolability is characteristic for white populations

Table I The activity of glycolytic enzymes in erythrocytes of the patient and its family

Enzyme	Enzyme activity			
	patient	father	mother	sister
Glucose-6-phosphate dehydrogenase	0 3-0 4	3 65	3 70	5 00
Phosphohexoseisomerase	16 00	8 50	10 60	10 00
Phosphofructokinase	2.70	1 60	1 60	1 70
Aldolase	1 50	0 75	0 95	1 71

The activity of G6PD is given as µmoles of substrate min g Hb the activities of other enzymes as µmoles min/10^{10} erythrocytes

for variants Seatle, Loyola, Eyssen and Ramat-Gan [2, 3, 7, 10] However, in types Seatle and Ramat Gan the *affinity for the substrate is increased* In other types no kinetic values were determined The Caucasian type has similar K_M values for G6P and NADP as our variant, but it has higher electrophoretic mobility [6]

According to the nomenclature of WHO Standardization Committee the phenotype of the presented variant of glucose-6-phosphate dehydrogenase can be called Gd (–) Wroclaw

The parents of the patient show normal erythrocyte G6PD activity The results of the ascorbate-cyanide test and glutathione reductase inhibition test performed on erythrocytes of the mother fall within the normal range No decrease of NADPH generating capacity was found in red blood cells It is known that in heterozygous G6PD deficiency the lack of glutathione reductase inhibition by chromate is evident even if a small percentage of deficient cells are present [11] Among other relatives no cases of hemolytic episodes were reported

The activities of some glycolytic enzymes (phosphohexoseisomerase, phosphofructokinase and aldolase) were determined in erythrocytes of the patient his parents and sister The results are shown in table I The deficiency of G6PD is accompanied by the increased activity of phosphohexoseisomerase phosphofructokinase and aldolase Similar changes were found in another case of glucose-6-phosphate dehydrogenase deficiency [5]

Acknowledgements We would like to express our thanks to Mr J Szymański for his technical assistance

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Acta haemat 46 197-200 (1971)

Observations on Cell Proliferation in Human Myelocytes

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Key Words
Autoradiography
Cytochemistry
Myelocytes
Myelopoiesis

Abstract Myelopoiesis has been studied using quantitative cytochemical cytometric and autoradiographic techniques. The earlier speculation that normal small myelocytes were mainly in G_1 and early S has been confirmed. The distribution of promyelocytes and myelocytes between the different stages of interphase was normal in β thalassaemia major and acquired sideroblastic anaemia the disturbance in cell proliferation in these disorders being confined to erythropoiesis. The mean labelling index of promyelocytes and myelocytes in patients with a reactive neutrophil leucocytosis was 15% higher than the mean for normal marrow indicating a significant increase in proliferation. Myelocyte labelling indices above 50% were seen in megaloblastic haemopoiesis due to vitamin B_{12} and folate deficiency and were rarely encountered in other diseases.

The proliferating myelopoietic cells include the myeloblasts promyelocytes and myelocytes and of these the myelocytes are present in largest numbers In previous kinetic studies of neutrophil granulocytopoiesis, CRONKITE *et al* [1] separated myelocytes into 2 serially-connected cell populations (representing 2 successive cell cycles) on the basis of their size After incubation with tritiated thymidine (^{3}H TdR) for 30 min, large myelocytes had a labelling index of 37% and small myelocytes a lower labelling index of 14%. More recently, from an analysis of the rate of appearance of labelled metamyelocytes after a single injection of ^{3}H-TdR, CRONKITE and VINCENT [2] have argued in favour of considering the myelocytes as one cytological class in which most of the small myelocytes represent G_1 (post mitotic rest phase) cells. Two cell divisions were thought to occur in the myelocyte compartment in the normal 'steady state' and the ways in which myelocyte proliferation may be increased were dis-

cussed So far there has been little work on the proliferative capacity of myelocytes in states of increased myelopoiesis In the present study, the relation between the size of myelocytes and their position in interphase has been investigated using the technique of Feulgen microspectro photometry to measure the degree of DNA replication in individual nuclei In addition, this technique has been combined with ^{3}H-TdR autoradiography to study the extent to which proliferation in the promyelocyte-myelocyte pool is altered in disease

Methods

Bone marrow aspirates were incubated with 7–12 μCi ^{3}H TdR/ml (specific activity = 5 000 Ci/M) in heparinised Hanks solution at 37 °C for 30 min Marrow fragments were then smeared on glass slides air dried fixed in methanol for 10 min and stained by the May Grünwald Giemsa method

The distribution of promyelocytes and myelocytes in G_1 S and G_2 was determined by studying cell morphology DNA content and DNA synthesis consecutively in the same cell as described previously [8 11] G_1 represents post mitotic cells with a diploid (2n) content of DNA S represents cells synthesising DNA and G_2 represents pre-mitotic cells after completion of DNA replication with a tetraploid (4n) DNA content A few myelopoietic cells do not fit into this classification and appear to be arrested after a period in DNA synthesis such cells are called U cells

In some patients only the ^{3}H TdR labelling index of promyelocytes and myelocytes was studied For this purpose the position and type of all myelopoietic cells in a selected area of the slide was recorded on a photographic map The May Grünwald Giemsa stain was then leached out the slide re stained by the Feulgen method and cells in DNA synthesis detected by autoradiography as described previously [8 10] By this sequence of operations cell morphology was studied in well stained smears prior to autoradiography and the difficulty in adequately staining myelopoietic cells through an autoradiograph was avoided.

For the determination of cell area, photographic negatives of May Grünwald Giemsa stained myelocytes were projected to a constant magnification and the outline of each cell drawn on paper The weight of the paper enclosed by each image was used as a measure of the area of that cell Nuclear areas were calculated from mean nuclear diameters which were measured after Feulgen staining using an image splitting eyepiece micrometer (Vickers Instruments Ltd)

Results

In figures 1 and 2 the DNA contents of normal myelocytes are plotted against cell and nuclear areas respectively Cell and nuclear sizes tend to be larger at higher DNA values The correlation coefficients (r) for fig

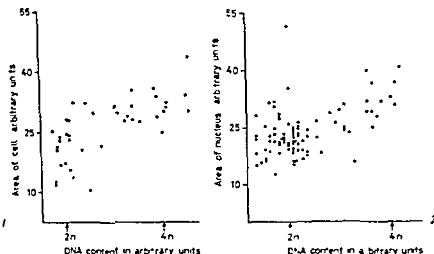


Fig 1 Relation between cell size and DNA content in the myelocytes in a normal marrow The open circles represent cells not labelled with ^{3}H TdR and the solid circles represent cells in S (r = 0 69)

Fig 2 Relation between nuclear size and DNA content in the myelocytes in a normal marrow (r = 0 60)

ures 1 and 2 are both statistically significant being 4.3 and 5 4 times their respective standard errors

Table I shows the percentage distribution of promyelocytes and myelocytes in the different stages of interphase in acquired sideroblastic anaemia, β thalassaemia major and untreated iron deficiency anaemia This is essentially normal in all 3 conditions The values for normal marrow and pernicious anaemia are calculated from the results of WICKRAMASINGHE and PRATT [11]

The ^{3}H-TdR labelling indices of promyelocytes and myelocytes in patients with a reactive neutrophil leucocytosis due to infection or neoplasia are shown in table II The minimum duration of the leucocytosis varied between 5 and 20 days. The mean value for the patients with a leucocytosis is higher than the mean for normal marrow

Table III shows the ^{3}H-TdR labelling indices of promyelocytes and myelocytes in a selection of patients with a variety of haematological diseases. All 4 patients with β thalassaemia major were taking 15 mg folic acid daily

Table I Distribution of promyelocytes and myelocytes in the various stages of interphase

Diagnosis	Percentages				Number of nuclei assessed
	G_1	S	G_2	U	
Acquired sideroblastic anaemia					
(3 cases)	67	30	3	0 4	464
Thalassaemia major (1 case)	78	20	2	0.5	192
Iron deficiency anaemia (2 cases)	61	35	4	0 2	496
Pernicious anaemia (5 cases)	38	57	4	1 5	1,168
Normal (3 cases)	61–70	25–34	3–5	0–1 2	653

Table II ^{3}H TdR labelling index of promyelocytes and myelocytes in patients with a reactive neutrophil leucocytosis and increased myelopoiesis

Haematological status	Blood neutrophils per μl	Number of subjects	Labelling index %		
			range	mean	2 SD
Reactive leucocytosis	9 000–17,500	6	38 2–56 0	45 1	2.6
Normal	2,500– 7 000	6	20 1–34 0	30 0	2 7

Myelopoiesis was also studied in a patient with megaloblastic haemopoiesis due to folate deficiency The haemoglobin level was 8 0 g/100 ml and the neutrophil count 1,000/μl on the day of the marrow aspiration In this patient, the ^{3}H-TdR labelling index of the promyelocytes was 67%. The myelocytes and giant metamyelocytes had labelling indices of 85 and 80% respectively

Discussion

From figures 1 and 2, it is clear that cell and nuclear size increases as a myelocyte progresses through interphase The separation of myelocytes into large and small on the basis of cell size is therefore, not justified In such a classification, small myelocytes are mainly G_1 and early S cells and large myelocytes include the majority of late S and G_2 cells The wide range of cell and nuclear areas observed at each DNA value is not surprising because of the non-linearity of the relation between area and

Dem n t at on of en ym a t vit es The water used was d st lled and de on zed a et ne was re l st lled Before being incubated the t ssue preparat ons were in c l l acet ne for 5 m nutes to rem ve l p ds and then in ? changes of lle l 0 l M phosphate sal ne p_H 5 for a total of 5 m nutes to remove water luble en l gen us sul strates

a) S me sect ns were then incubate l to demonstrate the l cat on and legree of v ty of Δ^5 3? l y lroxystero l dehy lrogenase an enzyme occurring early in the for stero l h rm ne b synthes s (*Samuels* 1960 *Rub n et al* 19 3 *Deane &* 19) Tl e me l um is g ven in Tal le ? The control med um was s m lar except at it lacked DHA Cenerally 4 t 7 c verslips were placed in a Columl a sta n ng r and incul at ns were carr e l out for l h and 3 h at 37 C.

Table ?

pos t on f me l um f r demonstrat ng tl e activity of Δ^5 3? bydroxysteroid dehy lr genase

	Amount
l uffere l w h 0 l M phosphate p_H 5	4 0 ml
l um (NBT S gma) l mg ml H_2O	2 0 ml
one (DHA S gma) 0 5 mg ml a etone	0.5 ml
ne d nucle t de (NAD or DPN Boehringer) mg ml H_2O	1 0 ml

t vity f NADH tetraz l um reductase otherw se known 0 ml of me l um conta ned buffer sal ne and NBT as PNH) Incul at ons usually ran f r ? t h

u tase act v ty the med um conta ned 5 mg generally lasted l h Th s enzyme may be con u phosphate pathway wh h is presumably in & *Dorfman* 19? *Deane et al* 19?)

were washed f xe l in ethanol formal n M unt ng Me l um



Day 15

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Pernicious anaemia (5 cases)	38	57	4	1 5	1,168
Normal (3 cases)	61–70	25–34	3–5	0–1 2	683

Table II ^{3}H TdR labelling index of promyelocytes and myelocytes in patients with a reactive neutrophil leucocytosis and increased myelopoiesis

Haematological status	Blood neutrophils per μl	Number of subjects	Labelling index %		
			range	mean	2 SD
Reactive leucocytosis	9,000–17,500	6	38 2–56 0	45 1	2 6
Normal	2 500– 7 000	6	20 1–34 0	30 0	2 7

Myelopoiesis was also studied in a patient with megaloblastic haemopoiesis due to folate deficiency The haemoglobin level was 8 0 g/100 ml and the neutrophil count 1,000/μl on the day of the marrow aspiration In this patient, the ^{3}H-TdR labelling index of the promyelocytes was 67% The myelocytes and giant metamyelocytes had labelling indices of 85 and 80% respectively

Discussion

From figures 1 and 2, it is clear that cell and nuclear size increases as a myelocyte progresses through interphase The separation of myelocytes into large and small on the basis of cell size is, therefore, not justified In such a classification, small myelocytes are mainly G_1 and early S cells and large myelocytes include the majority of late S and G_2 cells The wide range of cell and nuclear areas observed at each DNA value is not surprising because of the non-linearity of the relation between area and

the characteristics of myelocytes soon after the promyelocyte mitosis However, the possibility that some promyelocyte myelocyte transitions occur at all stages of interphase cannot be excluded If this were to happen, some promyelocytes and myelocytes would belong to the same cell cycle Furthermore, the morphological distinction between promyelocytes and myelocytes is not clear cut, there being a gradual transition between these 2 cell types For the above reasons, the results for promyelocytes and myelocytes have been pooled together in this paper As there were significantly fewer promyelocytes than myelocytes in all the patients studied, the labelling index of the promyelocyte myelocyte pool largely reflected the myelocyte labelling index.

In pernicious anaemia, labelling indices between 52 and 78% have been reported for the myelocytes [11] In the present study the labelling index of myelocytes in a patient with macrocytic anaemia due to folate deficiency was 85% Apart from a single patient with a reactive leucocytosis (neutrophils 17,500/μl) such high values were not encountered in a wide spectrum of other diseases studied (tables II and III) It is likely that the high myelocyte labelling indices in megaloblastic haemopoiesis result from a prolongation of DNA synthesis rather than a shortening of interphase

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Table II Duration from the time of diagnosis of chronic myeloid leukaemia till the onset of the conversion

Months	Number of cases	
6	1	
6–12	3	
12–18	6	
18–24	10	< 2 years = 20
24–30	4	
30–36	3	
36–42	2	
42–48	2	2–4 years = 11
48–60	2	
> 60	2	> 4 years = 4
Mean = 27		

Table III Treatment received by the patients with CML

Form of treatment	Cases of CML converted to AML	All cases of CML
No treatment	4	28
Busulphan	18	150
Splenic irradiation	6	31
Busulphan + splenic irradiation	7	16

Table IV Size of the spleen at the time of the conversion

Size in cm below the left costal margin	Number of cases
No enlargement	2
3	2
3– 6	3
6– 9	14
9–12	10
>12	4

Table I General health of the patients at the time of the conversion

	Number of cases
Very good	5
Good	17
Fairly good	9
Bad	4

Table II The main clinical manifestations ushering in the conversion

	Number of cases
Fever	10
Lassitude	5
Fever and lassitude	15
Bleeding	5

b) A fall in WBC (6 cases) from 28,000 to 6,000, from 32,000 to 8,000, from 44,000 to 8,000, from 56,000 to 9,500, from 52,00 12,000, from 62 000 to 11,000

c) A sudden fall of platelets (2 cases) from 200,000 to 20,000, f 250,000 to 15,000

d) A rise in WBC (3 cases) from 12,000 to 45,000, from 22 000 180 000, from 32,000 to 190,000 There was no concomitant rise of t myeloblasts at the peripheral blood

In all cases of the above paragraphs a–d, the typical picture of acu myeloblastic leukaemia at the peripheral blood appeared 1–3 month following the main haematological features, whilst during this period the bone marrow was typical of AML.

e) A rise of myeloblasts in the peripheral blood without a concomitant rise of the number of leucocytes (5 cases) from 3% to 80%, from 5% to 85%, from 1% to 45%, from 2% to 65%, from 5% to 85%

f) A rise of myeloblasts with a considerable rise in the WBC (8 cases)

Discussion

BOUSSER et al [1] reporting 22 cases of acute myeloblastic crisis found a median 18 months between the diagnosis of CML and the onset

Table 171 The main haematological features at the time of the conversion

	Number of cases
Fall of Hb and Ht	11
Fall of WBC	6
Fall of platelets	2
Rise of WBC	3
Rise of myeloblasts	5
Rise of WBC and myeloblasts	8

of the acute myeloblastic crisis CATTAN *et al* [2] reviewing 29 patients found that the acute conversion occurred at any time between three weeks and 7 years (median 26 months) after the apparent onset of CML. During the course of the basic illness, 3 received no treatment, 21 received chemotherapy, 1 splenic irradiation and 4 received a combination of chemotherapy and splenic irradiation According to the same authors the acute leukaemic syndrome was heralded by fever in 8, by bone pains in 4, by haemorrhagic manifestations in 4 and by the appearance of blasts at the peripheral blood in 9 cases MORROW *et al* [5] point out that amongst patients with chronic myeloid leukaemia in the terminal phase within 14 days before death, clinical and haematological changes were diagnostic of acute leukemia in 15, suggestive of acute leukaemia in 3 and indeterminate in 3 No correlation existed between preceding antileukaemic therapy and the onset of the terminal phase

The conversion of CML to AML can occur at any time during the course of the disease In our series it took place within the first 2 years from the time of diagnosis in 20 cases, during the third and fourth year in 11 cases whilst in 4 it occurred after the completion of the fourth year

In the majority of cases the conversion was not ushered in by the typical haematological features of AML. Thus in 22 of the 35 patients of our series the main haematological features at the onset of conversion were anaemia or a fall in the WBC or of the number of platelets As these features may also signify marrow aplasia induced by treatment either by busulphan or irradiation, a bone marrow examination should be carried out at the first appearance of any of the haematological features cited above

tic leukaemia and died 3 days after an unsuccessful attempt to obtain a living child by Caesarean section. She was not included in the trial with all other 10 patients mentioned. In addition, 2 patients with myeloblastic leukaemia, developing during the course of myelocytic leukaemia, were included in the study. The myelocytic phase in both of these had been less than 4 months.

The course of induction treatment consisted of 6-MP, 500 mg *per os* daily, divided onto 3 doses for a period of 5 days beginning as soon as the diagnosis was confirmed. In cases with a bleeding tendency, prednisolone at 40 mg *per os* daily was given. Further treatment was bases on a maintenance dose of 6-MP, 50–150 mg *per os* daily, up to tolerance. Antibiotics and blood transfusions were given to all patients, as well as thrombocyte concentrate to control bleedings when needed.

The evaluation of the remissions was made according to the criteria established by the Midwest Co-operative Chemotherapy Study Group [5]. Survivals were calculated from the day of diagnosis.

Results

The results of the treatment in the form of remissions, survival, and causes of death in the series are given in table I. In none of the patients could the remission be regarded as complete, but partial remissions were obtained in 6 of the 12 patients (50%). When comparing the results at various ages, the treatment resulted in a partial remission in 4 of 6 patients under 50 years of age, and in only 2 of 6 over 50.

Table I. Adult patients with acute leukaemia treated with 6-MP at high dosage

Case No	Age and sex	Results	Survival, days	Cause of death
1	48 F	failure	10	haemorrhage
2	51 M	failure	10	haemorrhage
3	58 F	failure	16	haemorrhage
4	69 F	failure	17	septicaemia
5	22 M	failure	34	septicaemia
6	77 F	failure	50	haemorrhage
7	73 M	PR	85	septicaemia
8	25 M	PR	117	septicaemia
9	68 M	PR	134	septicaemia
10	49 M[1]	PR	180	haemorrhage
11	16 F[1]	PR	195	haemorrhage
12	18 M	PR	245+	haemorrhage

[1] Cases with acute leukaemia developing during the course of chronic myelogenous leukaemia. PR = partial remission.

The 50% survival of the whole group was 50 days. Again, more advanced age was associated with a poor prognosis. The median survival period was 117 days for the 6 patients under 50, but only 17 days for the 6 over 50. An investigation as to the causes of death reveals a high rate of early deaths resulting from haemorrhagic complications among older patients.

Discussion

The reports on the treatment of acute leukaemia over the last few years have given the general impression that the best results evaluated by the frequency of remissions have been parallel to a high degree of myelotoxicity. On the other hand, the dosage of 6-MP in the maintenance treatment schedules is at the level earlier used in the initial phase of treatment. The result of the present series shows that when using 6-MP in the treatment of acute leukaemia in adults the frequency of remissions cannot be improved by increasing the dosage up to a level of severe myelotoxicity. This is, however, the experience of only single 5-day induction courses and does not exclude the possibility of better results by 2 or 3 repeated courses which are usual in the new combination schedules [2]. The fact that as many as half of the patients of the present series were older than 50 is also a factor contributing to the poor result [6, 8].

When introducing cytostatic treatment for acute leukaemia at myelotoxic level in any hospital, practical difficulties always exist in the treatment of complications. This is especially apparent in the use of thrombocyte concentrates to control bleedings. The high frequency of fatal bleeding among the early deaths may in part reflect the price to be paid in gaining experience in the treatment of these complications.

As a recent report on the use of a combination of cytostatics in the treatment of acute leukaemia in adults has been very promising [2], further trials with 6-MP as a single drug are hardly justified. It seems likely that the main value of 6-MP in the treatment of acute leukaemia is centred on the maintenance of remissions.

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Acta haemat 46 211-220 (1971)

Decreased Erythrocyte Deformability in Cholestatic Jaundice

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Abstract The anemia in experimental cholestatic jaundice has been studied in rabbits. A decreased erythrocyte deformability has been found in bile duct ligated animals and failed to be traceable in sham-operated ones The deteriorated deformability of the red cells in a first step is caused by the action of surface active substances and secondly by other factors as a possibly deranged cell metabolism Both factors will deteriorate the flexibility and thus the erythrocyte life span

Key Words
Erythrocyte shape
Cholestasis

Although a considerable number of investigations has been made into the many alterations of the red cell in hemolytic disorders, the real factors which limit the life span of the erythrocytes are poorly understood There is for instance, a shortened life-span of the erythrocytes in the anemia in cholestatic jaundice [1] and a decreased osmotic fragility [2] Auto-antibodies and splenic sequestration are in most cases less important [3] whereas low levels of red cell reduced glutathione suggest a metabolic defect [4] But it is not yet clear in which way these factors, not directly hemolytic acting will shorten the life-span of the erythrocytes

In this paper an attempt has been made to explain the increased destruction of red blood cells in cholestatic jaundice by the action of bile on the mechanical deformability of the erythrocyte

The well known erythrocyte deformability allows the passage of an 8 μm red cell through a 4 μm capillary without its being sequestrated The mechanical deformability and hence the capillary flow substantially depend on the cell shape [5] Since the cell membrane has little stretchability, the erythrocyte can only

adapt a streaming profile *if a large surface area surrounds a small intracellular volume* The biconcave shape of the normal red cell provides an optimal surface area volume ratio [6–8]

The importance of the biconcave shape becomes obvious from the mechanical properties of a spherocyte the surface area of a sphere must increase in size at any deformation If the membrane of this sphere, however, is not stretchable the result will be a rigid corpuscle

The surface area volume ratio of the normal biconcave erythrocyte seems to provide an undisturbed capillary passage for the red cell Therefore, any alteration of this shape factor will affect the microcirculation with direct consequences either to the red cell itself or, in many respects, to the total circulation From previous results it is well known that surface active substances (SAS) will cause the shrinking of the red cell membrane and thus the deterioration of the normal shape factor Bile is known to act as such an SAS A rapid result of the addition of small amounts of bile to freshly drawn blood, is a sphere with shrunken membrane *normal volume* and decreased deformability [9 10]

The increase by swelling of the cellular volume produces a second possibility to alter the normal shape factor of the erythrocyte [6 10] This will result in a *decreased* osmotic resistance, whereas the SAS treated cell is a normovolemic one Because the swollen cell has less surplus membrane due to the increased volume the ability to adapt to an optimal streaming profile will be reduced

The available data show that, *in vitro*, bile as an SAS will immediately deform the biconcave erythrocyte into a normovolemic sphere with normal osmotic resistance whereas, *in vivo*, cholestatic jaundice will result in a *decreased* osmotic fragility of the red cell In this paper, an attempt has been made to find the key to this disagreement

Methods

The common bile duct of 8 rabbits weighing from 2 8–3 6 kg were ligatured under nembutal ether anesthesia and 6 rabbits were sham operated Blood samples were drawn by puncture of the ear vein before the operation and every 2nd day after it The total blood loss was in the region of 15 ml The animals were killed on the 7th–9th day after the operation by a blow on the back of the neck Hemoglobin microhematocrits and total serum bilirubin were determined The density of the erythrocytes was ascertained by the Phthalatester method [11]

The mechanical deformability of the erythrocytes was investigated by a previously described method [12 13] based on the following principle the centrifugation at 32 *g* of red cells in glass capillaries of 50 mm length and an inner diameter of 2 mm Normal erythrocytes are assumed to adapt an optimal streaming profile at these *g* values whereas less deformable red cells will retain their original shape No packing of the red cells occurs due to the length of the glass capil

laries As a result of the different flexibility the less deformable erythrocytes have a greater flow resistance and thus a lower migration speed The difference between biconcave erythrocytes and crenated spheres reaches the ratio 5 1

The resistance of the red cell to hypotonic NaCl was determined at room temperature After 1 h the solution was centrifuged and photometrated

For the determination of intracellular Na^+ and K^+ with the flame photometer the erythrocytes were washed once in an isosmolar cholin-chloride solution

Results

Shape The normal biconcave shape of the rabbit erythrocyte is changed after bile duct ligature Up to 30% of the cells in the wet glass preparation become crenated spheres until the 3rd postoperative day These spheres are regularly shaped whereas on the 7th–9th day after bile duct ligature fragmentation and every red cell abnormal shape may appear

Resistance to hypotonic NaCl During the first 3–4 days after bile duct ligation the osmotic resistance of the red cells is unchanged whereas a decreased resistance becomes significant ($p = 0.01$) from on the 5th day the more pronounced the longer the cholestasis was lasting

Deformability The above-described method shows the normal erythrocyte flexibility expressed as migration speed at centrifugation with 32 g as 21 mm 30 min The sole narcosis and operation stress diminishes the migration speed on the 1st postoperative day to 12 mm 30 min ($p = 0.01$) in both unl groups Whereas there is a quick recovery of red cell flexibility in the sham-operated animals the migration speed remains lowered in cholestatic jaundice (fig 2)

Hemoglobin and *hematocrit* did not change in sham-operated animals In jaundiced rabbits the hemoglobin content decreases to 10 5 g% Rabbit erythrocytes have a normal middle density of 1 1100 No significant alteration occurred but a more level course of the graph was a constant finding

Electrolytes From the normal Na^+ content of the erythrocytes of 56 mg% and K^+ of 350 mg% there was a change after 9 days to 64 mg% for Na and 280 mg% for K^+ ($p = 0.05$) (table 1)

Histology Microscopic examination of H and E stained liver slices made apparent a sometimes excessive dilatation of intrahepatic bile ducts but no further important pathologic finding There was, in particular, no sign of an infectious hepatitis



Fig 1 *a* Wet glass preparation of normal rabbit erythrocytes *b* red cells with 0 3% and *d* 0 7% of bile *in vitro* and in comparison red cells *e* 3 and *g* 9 days after common bile duct ligation The shape of the spherocytes of figure *b* and *e* can be normalized to nearly biconcave cells by the addition of chlorpromazine (*c* resp *f*)

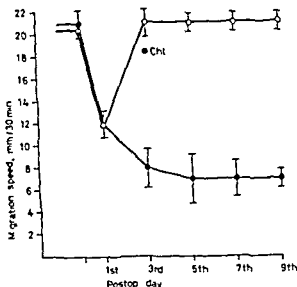


Fig 2 Deformability of rabbit red cells during centrifugation with low g values. In sham-operated animals (○—○) the flexibility remains unchanged except a short drop on the 1st postoperative day, whereas after bile duct ligation erythrocytes keep a highly significant lowered flexibility (●—●). In the 1st days chlorpromazine (Chl) will improve the deformability, but usually will be ineffective at the 7th or 9th day

Discussion

Changes in shape and deformability of the red cell by the action of surface-active substances in vitro. The *rapid* deformation of the shape of the normal biconcave red cell into a crenated or smooth sphere can be mediated by the action of several anionic and non-ionic substances, whereas cationic substances will result in a cup-like stomatocyte [14]. Both groups of SAS act on the erythrocyte membrane in an opposite way. One group can be displaced by the other, the whole process being reversible. Thus the membrane will be shrunk for some 20–30% by bile (belonging to the group of anionic and non-ionic substances) and will be stretched by chlorpromazine (group of cationic substances) [15] (fig 1b, c). During this process, the bile-produced spherocytes maintain a normal osmotic resistance (fig 4) and a normal critical hemolytic volume [10] indicating that no substantial loss of the cell membrane has

Table I

	Before operation	1st day	3rd day	5th day	9th day
Bilirubin mg%	0.4	2.3	2.7	3.2	4.6
Hemoglobin, g%	14.4	13.8	11.1	10.8	10.5
50% hemolysis, % NaCl	0.45		0.44	0.52	0.59
Spec density	1.1120				1.1095
Ery Na⁺, mg%	56±0.7				64±1.2
Ery K⁺, mg%	350±4.5				280±7
Ery migration speed mm/30 min	21±1.2	12±1.2	8±1.7	7±2.2	7±0.8

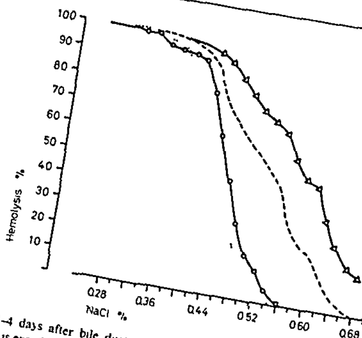


Fig 3 3–4 days after bile duct ligation ( ) the osmotic resistance of rabbit erythrocytes is exactly the same as in normal cells (○—○) whereas the deformability of the red cells by this time is strongly reduced. On the 5th to 9th day (- -) (-△-△-) a decreased osmotic resistance is significant.

occurred. Determined with the Coulter counter, the bile-shrunken cell has exactly the same volume as the biconcave erythrocyte [16]. The normal surface-volume ratio, however, has been affected and therefore the deformability decreases as has been pointed out in the introduction.

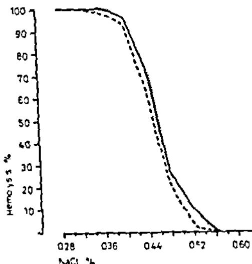


Fig. 4. No alteration of osmotic resistance to hypotonic NaCl occurs by the addition of 0.3% ( ) or 0.7% bile ( ) to normal erythrocytes

the migration speed in our 32 g centrifuge decreases from 22 to 9 mm/30 min [13].

The sphering process by anionic substances and the restoration of the shape by chlorpromazine are minute events: the cells change their form *rapidly* at the moment of contact with the SAS. This abrupt event shows that cellular metabolism will scarcely be involved in this process.

Surface-active substances in vivo. Figure 1 shows the shape of the erythrocytes on the 3rd (e) and 9th days (g) after bile duct ligature in serum suspended normal red blood cells (a). There is good evidence that the spherocytes which can be seen *in vivo* on the 3rd postoperative day are produced by a similar mechanism as in the cells which result from the addition of 0.3% bile (fig. 1b). The migration speed of the red cells on the 3rd postoperative day is restricted to 8 mm/30 min, the osmotic resistance is still unchanged, the shape can be restored by chlorpromazine (fig. 1f) and by this the migration speed increases to 18 mm/30 min (Chl *in* fig. 2).

The beneficial action of chlorpromazine upon the erythrocyte deformability and cell shape after bile duct ligature agrees with previous investigations, e.g. it is well established that chlorpromazine is able to

restore the shape of smooth and rigid spherocytes which are produced *in vivo* by the i.v. application of free fatty acids [17] or 2 μg/min/kg adrenaline [18]. In the same way chlorpromazine is able to return the shape and deformability of erythrocytes after severe burns to near-normal levels [19]. In all these papers, it has been supposed that on account of accumulating SAS in the plasma a shrinking of the red cell membrane takes place. The stretching effect of chlorpromazine interferes with this shrinking process in SAS.

The view that a plasmatic factor should be responsible for the changes in cell shape and erythrocyte deformability is assumed by convincing experiments, where normal, freshly-drawn erythrocytes were crossed with serum of jaundiced rabbits. The repeated result was a rapid sphering process, the migration speed decreasing from 22 to 10 mm/30 min.

POWELL [1] who examined the cause of hemolysis in jaundice, s... posed the conjugated bilirubin to be operable in patient's plasma, whe as conjugated bile acids, phospholipids and cholesterol are ineffective production of a hemolytic anemia. It should be accentuated, howeve that *in vitro* an increased hemolysis by bilirubin glucuronide takes plac only at abnormally high concentrations [20]. This contrast between the moderate bile induced hemolysis *in vitro* on the one hand, and the clear influence of low bile concentrations on shape and deformability of the red cells *in vivo* and *in vitro* on the other hand, should lead to the view that the reduced erythrocyte deformability and not the direct action of bile on the red cells may be correlated to the hemolytic anemia after bile duct ligature.

Red cells of lower deformability are thought to be less adapted to capillary flow [23] though it is not yet clear what happens in capillaries. It is conceivable that less deformable red cells will stick mechanically in the microcirculation [8]. Another opinion exists that during metabolic stress, which will be in process during an aggravated capillary fatal passage, changes of the erythrocytes occur [25].

Secondary changes of the red cells in the course of traceable anemia. The lower efficiency of chlorpromazine was a striking effect in the process of the experiment. After approximately 5 days the deformed cells, such as crenated or smooth spheres, cannot be altered into normal-shaped or normal flexible erythrocytes by chlorpromazine. It is obvious hat additional factors are acting upon the flexibility of the red cells he increased intracellular Na^+ and the reduced osmotic resistance at

the same time call to mind a derangement of the cellular metabolism, which leads to a swollen erythrocyte. In 3 examined cases, the glucose consumption of the erythrocytes had been reduced. From either investigation it is well established that ATP-depleted red cells are shaped as crenated or smooth spheres [26], for some reason on account of Ca^{++} binding to the cell membrane [27]. From this, the lower efficiency of chlorpromazine at this state of the experiment is well explicable: chlorpromazine can only improve a deranged shape which has been induced by SAS, whereas a metabolic defect will not be influenced.

Stress should be laid on the fact that in this secondary period, too, where SAS are less important, the first factor causing the production of anemia also may be the erythrocyte deformability, and not the deranged cellular metabolism or the decreased osmotic resistance *per se* [28].

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Platelet Defects in Post-Splenectomy Thrombocythaemia

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Key Words
Platelet aggregation
Platelet defects
Splenectomy
Thrombocythaemia

Abstract. A 43-year-old female, when subjected to splenectomy for obscure splenomegaly, gradually developed an increasing bleeding diathesis. About 20 months after splenectomy her platelets numbered 1.3 million/mm³ and showed complete absence of aggregation by ADP, epinephrine and nor-epinephrine but a normal reaction towards collagen; the activity of platelet factor 3 was also poor. After an inadvertent overdosage of myelo-suppressive therapy she went into a thrombocytopenic phase but the platelets readily recovered to a normal number and function on steroid therapy. The patient has been maintaining a normal platelet status since December 1969.

Splenectomy is usually followed by a symptomless temporary thrombocytosis [7, 8, 9, 23, 27, 28], but sometimes thrombocythaemia results [13, 15, 16, 22] that may be associated with thrombo-embolic complications [1, 4, 6, 10, 13, 20]. Though haemorrhage has been mentioned as a complication in postsplenectomy thrombocythaemia [18, 22], it is probably much less common. Literature on haemostatic defects responsible for bleeding state in post-splenectomy thrombocythaemia is very scarce. HAYES *et al.* [13] while discussing the problem in this context described 1 case where bleeding was the main complaint. However, their patient was also interpreted as having temporary deficiency of PTA (factor XI) that returned to normal after treatment of patient with alkylating agents. In a study of 16 patients with postsplenectomy thrombocytosis, MCCLURE *et al.* [17] found bleeding episodes in 2 patients but they thought that haemorrhage could be adequately explained by some factor other than a high platelet count and that bleeding had preceded the period of thrombocytosis.

The purpose of this paper is to communicate the unique aggregation abnormalities of platelets in addition to defective liberation of platelet factor 3 (PF 3) in a case of post-splenectomy thrombocythaemia with bleeding manifestations

Case Report

A female patient aged 43 years was subjected to splenectomy on 29 8 67 in the J J Group of Hospitals Bombay For 5 years prior to operation she experienced low grade fever off and on and since 1965 she felt a gradually enlarging lump in the abdomen In July 1967, she passed black tarry stool for about 3 days The pre operative clinical appraisal showed an enlarged spleen palpable 8 cm below the left costal margin There were no ascites nor any prominent veins over the abdomen Haemoglobin 14 7 g%, total leukocyte count 10 800 mm³, with neutrophils 66%, lymphocytes 28%, eosinophils 4% and monocytes 2% The blood film showed no abnormal leukocytes nor any monocytes with malarial pigment The red cells were normochromic normocytic and no malarial parasite could be demonstrated on repeated blood examinations The erythrocyte osmotic fragility was within normal range Erythrocyte sedimentation was 8 mm for the first hour (Wintrobe) Whole blood clotting time and bleeding time were 4 and 2 min 30 sec, respectively Platelets 270 000 mm³ of blood Aldehyde and antimony tests for Kala Azar were negative Urine and stool examination showed no abnormality Skiagram of chest revealed nothing significant

The immediate post operative period was uneventful After 3 months she had an attack of gross haematuria which cleared within 4 days In February 1968 she bit her tongue and bled profusely from the site for several hours Since April 1968 she started having frequent episodes of epistaxis and almost continuous oozing from her gums She also noted haematoma formation after mild injuries On 15.5 69 she was admitted in the Sir Sunder Lal Hospital Banaras Hindu University, and since then has been under our care

She was of average build with no anaemia or cyanosis Her gums showed obvious oozing of blood and her teeth were deeply stained with old blood There were no petechiae or ecchymoses Liver was palpable 2 cm below the right costal margin smooth and non tender She had a movable right kidney and i v pyelography showed mild distortion of its calyces

Haematological findings haemoglobin 14.5 g%, erythrocyte count 4 8 millions mm³ leukocyte count 12 600 mm³ with neutrophils 78%, lymphocytes 10%, eosinophils 10% and basophils 2% Late normoblasts were 6% of leukocytes and occasional fragments of megakaryocytes were also seen in the blood film Platelet count was markedly increased to 1 3 millions mm³ Bone marrow was hypercellular with marked megakaryocytic hyperplasia Regarding coagulation profile whole blood clotting time bleeding time clot retraction kaolin cephalin clotting time prothrombin time thromboplastin generation (employing cephalin [21] as a platelet substitute) were all within normal range Prothrombin consumption index was 15% (in our laboratory the normal is less than 15%) All these coagulation tests were done as described by BIGGS and MACFARLANE [3]

Table I. Post-splenectomy thrombocythaemia. Macroscopic aggregation of platelets

Product added	Concentration	Aggregation time, sec			
		May 22, 1969	Nov 21, 1969	Dec 30, 1969	normal range
Adenosine-diphosphate	10^{-4} M	no aggregation	32.2	12.0	11–18
	10^{-5} M	no aggregation	35.6	14.5	13–22
	10^{-6} M	no aggregation	36.4	17.6	16–24
Epinephrine	5.4×10^{-4} M	no aggregation	56.00	19.8	16–24
Norepinephrine	5.9×10^{-4} M	no aggregation	72.2	21.0	16–24
Collagen	-	21.0	20.8	21.6	18–24

Table II. Post-splenectomy thrombocythaemia. Reaction of platelets to kaolin

Low-spun plasma[1]	+ High-spun plasma	Kaolin time, sec		
		May 22, 1969	Nov 21, 1969	Dec 30, 1969
Patient	patient	58	33	31
Patient	normal	56	32	31
Normal	patient	30.6	31	32
Normal	normal	31	31.5	31.5

[1] Platelet count adjusted to 0.2 million/mm³.

The platelet studies were performed on May 22, 1969, 6 days after keeping the patient on only ascorbic acid, 500 mg/day. The platelet count was done by direct counting using Thoma's pipette and the improved Neubauer chamber, sodium citrate was used as a diluent. Macroscopic examination of platelet aggregation with adenosine diphosphate (ADP), epinephrine, norepinephrine and collagen was carried out at 37 °C by adding 0.1 ml of these reagents to 0.2 ml of platelet rich plasma. Microscopic examination was done on a coverslip preparation, intermittently for 60 min. Collagen suspension was prepared according to SPAET *et al.* [24]. Reaction of platelets to kaolin was studied according to HARDISTY *et al.* [11]. The results of platelet studies are shown in tables I and II.

The patient was put on Myleran® (busulfan), 4 mg/day and was discharged from the hospital on May 27, 1969, with instructions to have a haematological check-up done every week. However, since the bleeding symptoms of the patient gradually disappeared, she did not report to the hospital and continued taking My-

leran* till her gums started bleeding again She attended the hospital on July 23, 1969, and was found to have developed thrombocytopenia (platelet count, 48,000/mm³ and weakly positive tourniquet test) Prothrombin concumption index was 70% . She had acquired marked pigmentation of both hands arms and face Myleran* was immediately withdrawn and prednisolone (30 mg/day) was instituted The bleeding tendency disappeared as her platelet count returned to normal, a steady count is being maintained since November 21, 1969 The aggregating properties of the platelets also became normal by December, 1969 (table I) Steroids were gradually withdrawn by January, at which time her pigmentation also completely disappeared The last 'follow up' assessment was done on May 28, 1971 when all her haemostatic functions were within normal range

Since we were unable to get the histology report on the spleen removed at Bombay, we were intrigued about the obscure cause of splenomegaly The patient was admitted in September, 1970 Liver biopsy showed no significant histological changes

Discussion

The present patient had several interesting features The haemostat defect was moderately severe, with impaired release of PF 3 from plat lets by kaolin treatment and striking aggregation abnormalities of plate lets There was complete absence of platelet aggregation by adenosine di- phosphate, epinephrine and norepinephrine, but a normal response with collagen The prothrombin consumption was initially considered at the upper limit of normal but its considerable improvement after the return of platelets to normal number suggests a mild initial abnormality of this function also The bleeding time and clot retraction were well within the normal range

This combination of platelet defects seemed to us unique and undescribed in the literature However, at the time of this paper, a report by SPAET *et al* [25] has appeared describing similar aggregation abnormalities of platelets but with normal PF 3 release, in 3 patients with essential thrombocythaemia These workers also observed reduced uptake by platelets of serotonin labelled with ^{14}C and possibly reduced platelet adenosine-diphosphatase activity

The platelet defects described in our patient differ from the previously described functional defects The thrombasthenic platelets fail to aggregate with the usual aggregating agents including collagen and also have poor clot retracting activity The patient had normal clot retraction and her platelets reacted normally to collagen This normal reaction of collagen is probably responsible for the normal bleeding time

The other aggregation abnormalities of platelets associated with a bleeding diathesis are constituted of an anomalous reaction to epinephrine and collagen but with normal ADP induced aggregation [12, 14, 21]. CAEN *et al* [5] described the familial occurrence in 3 individuals of similar platelet abnormalities. In the patients of WEISS [26] platelets showed reduced PF 3 availability as well as poor aggregation by collagen and abnormality in adhesiveness. These abnormalities were attributed to a defect in the release of intrinsic platelet adenosine-diphosphate.

It has become important to exclude the exogenous effect of certain drugs which can induce platelet aggregation abnormalities. The notable examples are aspirin, phenyl butazone, some antihistaminics and tranquilizers [19, 29]. The patient was put on ascorbic acid exclusively with no other drug for 6 days before the initial platelet studies were undertaken.

The patient showed a remarkable clinical and haematological response to treatment. She has been under follow-up for more than 2 years and has been completely asymptomatic since November, 1969. As the effects of overdosage of Myleran® gradually disappeared and platelet number returned to normal, the PF 3 release and aggregation properties became normal. Aggregation by epinephrine became normal a little later than the reaction by adenosine-diphosphate. It is of interest that the patients of SPAET *et al* [2] had more platelet abnormality towards epinephrine as compared to ADP, and in one of their patients the epinephrine defect persisted when the ADP response had been largely corrected following myelosuppressive therapy.

Acknowledgement. We are grateful to Prof. K. N. UDUPA, Director and Professor of Surgery, for kindly referring the patient to us. We extend our gratitude to Dr. J. M. GUPTA, Professor of Pathology, for kindly providing the facilities for investigations, and to Shri R. M. MISHRA, technician, for technical assistance. We are thankful to Dr. T. R. MOTWANI, J. J. Group of Hospitals, Bombay, who operated on the patient and whose records have been used in the present report.

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Materials and Methods

We took a quantity of blood equal to 1% of the body weight, with cardiac puncture, under aether anaesthesia, from R Amsterdam rats of both sexes, weighing 220–280 g (according to informative examination carried out on 10 animals, the initial haematocrit value of 55% decreased to 23% 6 h after the taking of blood), but the number of thrombocytes did not change substantially

After 6 h the rats were exsanguinated and their serum administered i p to the members of a group of mice consisting of 5 animals (so-called 'mouse test' [7]) The result of the experiment was considered to be positive, if the average increase in the thrombocyte count of recipient mice reached or exceeded 30%, and the change occurred in all animals, or was below 30% in only one Splenectomy, adrenalectomy and hypophysectomy was performed 48–72 h, nephrectomy or ligation of ureter carried out 2–18 h before the transfer of serum or exsanguination.

Of the solution prepared from thrombokinase (Geigy) tablets, coagulating normal plasma within 15 sec, 0.5 ml/250 g body wt. was administered in the form of venous injection In nephrectomized animals the injection was carried out 2 h after the operation The thrombocyte count showed after 3 min a decrease from the original 1,000 000 to 20 000 – 110 000 The serum transfer – at the maximal activity observed during previous experiments – was carried out after 24 h.

For the thrombocyte count we used the direct phase contrast microscopic method described by FISCHER and GERSTER [2]

Results

In the mouse test employed the serum of normal rats did not cause any significant changes in the number of blood platelets, thus being negative, whereas the serum obtained 6 h after exsanguination was positive (according to our examinations the maximal thrombopoietic serum activity in rats may be noted 6 h after exsanguination, decreases after 12 h and can no more be observed after 24 h)

After splenectomy, adrenalectomy and nephrectomy the serum of rats is, like that of normal rats, negative in the mouse test The serum obtained 6 h after exsanguination, which was carried out 48–72 h after splenectomy, adrenalectomy and hypophysectomy, increased the thrombocyte count in recipient mice by 40–60%, i e it was positive, thus the extirpation of the above-mentioned organs does not suspend the appearance of the exsanguine thrombopoietic serum factor

After bilateral nephrectomy the serum of rats has no thrombopoietic activity The serum, obtained after 6 h, of animals exsanguined 2 or 18 h after bilateral nephrectomy, is negative, whereas the serum of rats

Acta haemat. 46: 228–231 (1971)

Study on the Development of Posthaemorrhagic Thrombocytosis in Rats

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Key Words
Thrombocytosis
Thrombopoietic factor

Abstract. After one or two days blood loss causes thrombocytosis and in the serum of exsanguined experimental animals a thrombocytosis producing factor appears. Splenectomy, adrenalectomy and hypophysectomy do not suspend the appearance of the serum factor. After exsanguination following nephrectomy by 2–18 h the serum factor inducing thrombocytosis does not appear, whereas animals with ureter ligation react like those not operated upon. During the phase of isolated thrombocytopenia produced by intravenous administration of thromboplastin the serum of nephrectomized animals shows a thrombocytosis producing activity.

Following a latency of one or two days blood loss causes thrombocytosis. The maximal increase in the number of blood platelets may be expected on the fifth day, which exceeds the initial value by 70–200% depending on the degree of bleeding and on the species. We proved in our earlier study [4] that few hours after the blood loss the serum of the experimental animal has a thrombopoietic activity, which when transferred to the recipient animal, causes thrombocytosis with a maximum on the fifth day. Posthaemorrhagic thrombocytosis is taking place through the proliferation of megakaryocytes [5, 6] and therefore it may be assumed that a 'poietin' is responsible for the increase in the number of blood platelets. Up to the present, however, the place of origin of the factor producing thrombocytosis is not clear.

With our present experimental series we endeavoured to identify the place of origin of the humoral substance producing thrombocytosis by the determination of thrombopoietic serum activity produced in rats through exsanguination performed after the extirpation of various organs.

Materials and Methods

We took a quantity of blood equal to 1% of the body weight with cardiac puncture, under aether anaesthesia from R Amsterdam rats of both sexes, weighing 220–280 g (according to informative examination carried out on 10 animals, the initial haematocrit value of 55% decreased to 23% 6 h after the taking of blood) but the number of thrombocytes did not change substantially

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After splenectomy adrenalectomy and nephrectomy the serum of rats is like that of normal rats negative in the mouse test The serum obtained 6 h after exsanguination which was carried out 48–72 h after splenectomy adrenalectomy and hypophysectomy increased the thrombocyte count in recipient mice by 40–60% i e it was positive, thus the extirpation of the above mentioned organs does not suspend the appearance of the exsanguine thrombopoietic serum factor

After bilateral nephrectomy the serum of rats has no thrombopoietic activity The serum obtained after 6 h of animals exsanguined 2 or 18 h after bilateral nephrectomy is negative, whereas the serum of rats

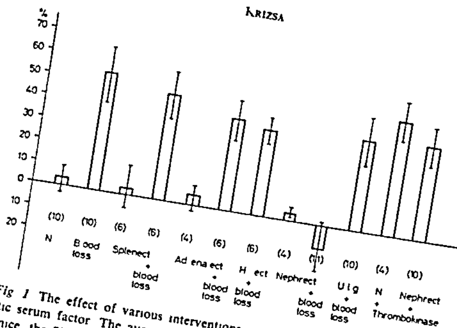


Fig 1 The effect of various interventions on the appearance of the thrombopoietic serum factor The average of changes in the thrombocyte count of recipient mice the SD as well as the number of donor rats in each group is indicated N = normal Splenect = splenectomy Adrenalect = adrenalectomy H ect = hypophysectomy Nephrect = nephrectomy U lig = ureter ligature

after exsanguination after bilateral ligation of the ureter is positive, like that of unoperated animals (the RN of nephrectomized animals, as well as of animals with ligation of the ureter did not show any appreciable difference)

The serum of thrombocytopenic rats treated with thrombokinase caused an increase of 54% in the thrombocyte count in the mouse test The serum of nephrectomized animals treated with thrombokinase yielded on the fifth day a significant increase of blood platelets in mice, i e it was positive meaning that the increase of thrombopoietic activity produced by thrombocytopenia may develop also in nephrectomized animals

Discussion

Since the investigations of KELEMEN *et al* [3] it has been known that similarly to erythropoietin a humoral factor plays a role in the regulation of thrombocyte production But its place of origin is not yet cleared The study of the role of the kidneys in this connection is not

easy. In this connection only the data of DE GABRIELE and PENINGTON [1] are known in the literature, who observed that the thrombocyte count in animals did not decrease for 4 days after nephrectomy, and cytopenia became gradually moderate for 3 days, during the period of analysis after the use of antithrombocyte serum. Concerning these investigations, it may be remarked that the action of 'poietin' on the periphery can be measured only after 48–72 h, thus the thrombocytes counted by these authors originated from the giant cells differentiated already before nephrectomy.

According to our data the presence of the kidney is necessary for the appearance of the posthaemorrhagic thrombopoietic factor. Nevertheless, increased thrombopoietic activity with significant thrombocytopenia may be produced also in the nephrectomized animals. It is possible that posthaemorrhagic thrombopoietic activity differs qualitatively from the increased thrombopoietic activity developing as a consequence of thrombocytopenia, but it might be that the kidney (erythropoietin?) has a permissive role in the increase of posthaemorrhagic thrombopoietic activity.

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Acta haemat 46 232–241 (1971)

Hereditary Elliptocytosis Associated with Beta-Thalassaemia and a Variant of Rh (D)

A Study in a Sinhalese Family

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Abstract A Sinhalese family with hereditary elliptocytosis is been described Three of the members had thalassaemia trait and 2 had CD^ue chromosome There was no abnormality due to the elliptocytosis nor to the presence of the thalassaemia trait Both genes had not interacted The osmotic fragility was markedly decreased in 2 members before and after incubation Two members did not show any haptoglobin bands

Key Words
D^u blood group
Elliptocytosis
Thalassaemia

Hereditary elliptocytosis is an uncommon disorder and its inciden in Ceylon is not reported Thalassaemia, however, is not uncommo [1–3] Both β- and α-thalassaemia are known to exist, while instances o interaction of thalassaemia with Hb E are also described [3, 4] Multipl inherited erythrocytic abnormalities have been known to occur in families Cohen *et al* [5], Cunningham and Vella [6] and Swarup *et al* [7] described the occurrence of hereditary spherocytosis with thalassaemia Beta-thalassaemia with hereditary elliptocytosis has also been reported [8–10] Hereditary elliptocytosis with Hb C [11], with Hb S [12] and with hereditary persistence of foetal haemoglobin [13] have been described

This is a study of a Sinhalese family whose members showed a combination of hereditary elliptocytosis β-thalassaemia trait and a variant D^u antigen in the Rh system

Methods

The methods used in the haematological investigations have been described elsewhere [4]. Electrophoresis of haemoglobin was carried out using paper at pH 8.6 (veronal) and 8.9 (tris buffer). Estimation of Hb A_2 was carried out on cellulose acetate strips [14]. Foetal haemoglobin was estimated by alkali-denaturation technique [15]. For demonstration of erythrocyte inclusion bodies brilliant cresyl blue vital staining was used. Haptoglobin types were determined by starch gel electrophoresis [16]. Screening test for G6PD deficiency was done by using brilliant cresyl blue dye decolorization test [17]. Total glutathione levels of the red cells were estimated by the modified nitroprusside method [18]. The variation in the agglutination reaction were further scored by titrating with 13 different incomplete anti Rh (D) sera free from anti C with the papain treated red cells of all the members of the family. One of incomplete anti D was obtained from a Cde/cde (R r) patient. Three Rh sera were from Ortho Diagnostics, USA, and the rest were locally prepared. All Rh sera had Rh(D) specificity except one which was anti DE.

Case Reports

Case 53 propositus. A Sinhalese male, aged 22 years, was admitted for investigation on 14.11.1969 with a history of fever of 4 days duration and an enlarged spleen. He had just returned from an area where malaria was endemic. He had been in good health prior to this.

Examination revealed a febrile individual. He had no other abnormality except 2 fingerbreadths palpable spleen below costal margin. The same evening he had a rise in the temperature with chills and rigor and was clinically thought to have malaria and was given anti malarial drugs, following which he remained afebrile.

Haemoglobin 10.2 g%, PCV 27%, MCV 92.5, MCHC 30.3%, reticulocytes 0.4%, WBC 9400/mm³, P 70%, L 26%, E 4%. Large number of elliptocytes (about 75%), mild aniso-poikilocytosis and a fair number of target cells (fig 1b). Paper electrophoresis revealed no abnormal haemoglobin. Hb A_2 was increased (3.5%). There was no increase in the alkali-resistant haemoglobin (1.33%). G6PD activity was normal and total glutathione level in the red cells was 30.0 mg%. No haptoglobin bands were detected in the serum on starch gel electrophoresis on repeated examination. Agglutination reaction with 2 routine anti Rh(D) sera gave slightly weaker reaction than in the mother (52) and the sister (54).

Diagnosis. Hereditary elliptocytosis and thalassaemia trait.

Figure 2 shows the family tree.

Case 51 father, aged 50 years, was asymptomatic. Haematological, biochemical and liver function tests are shown in tables I and II. Large number of elliptocytes and target cells were seen (fig 1a). Haemoglobin A_2 was increased (4.22%). The red cells gave strikingly weak positive to positive (+) agglutinations with 13 different incomplete anti D sera used routinely in the laboratory as compared to other members of the family (52, 53, 54) and control samples. Further, the titration

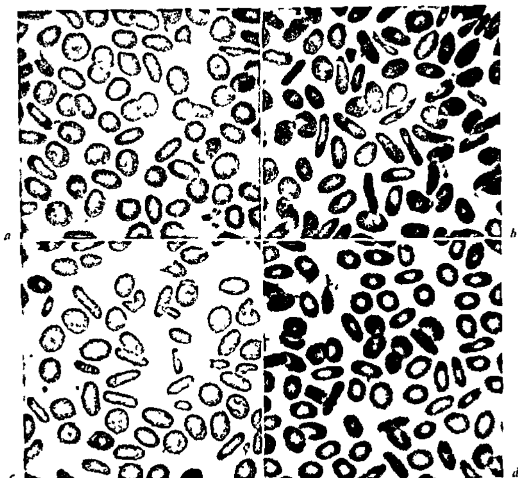


Fig. 1 Peripheral blood of father (S1), propositus (S3), sisters (S4, S5)

score with selected anti Rh(D) sera showed on the average poor score 7 with S1 cells, whereas *S2, S3 S4 R_1r and R_1R_1 controls gave 25, 20, 23, 30* and 35 score values respectively Control fresh cells showed higher scores The red cells of S1 (father) exhibited a variant of D and is designated as D^u, since the reactions and the scores were consistently weak as compared to other members of the family (S2 S3 and S4) The case therefore appears to be similar to one described by DUNSFORD [19] where his D subject consistently gave weak agglutination with most Rh sera used A same variant was found in his family

In few instances Cde(R) chromosome has been reported to suppress the expression of D thereby apparently behaving as a high grade D^u and may give weak reactions Such a possibility could arise if the S1 has an extremely rare genotype Cde/cDe(R Ro) But the ccd dee phenotype of S5 (sib) and CcD ee of S2 (mother) ruled out the possibility of S1 (father) being a rare type Cde/cDe(R Ro)

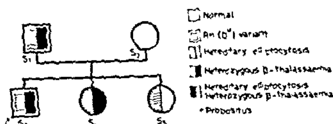


Fig. 2. Family pedigree showing hereditary elliptocytosis, β thalassaemia trait and Rh(D^u) variant

Diagnosis: Hereditary elliptocytosis, thalassaemia trait and Rh(D^u) variant

Case S2, mother, aged 40 years, had no abnormality. The bloodpicture showed mild hypochromia. Results of the other investigations are shown in tables I and II.

Diagnosis: Normal.

Case S4, sister, aged 24 years, had no symptoms but her mucous membranes were pale. Her spleen was not palpable. Besides moderate anemia (table I), the blood picture revealed a large number of elliptocytes, moderate anisopoikilocytosis, target cells, hypochromia and microspherocytes (fig. 1c). The haematological and biochemical data are summarised in tables I and II. Haemoglobin A_2 was increased (4.02%). No haptoglobin bands could be detected on starch gel electrophoresis.

Diagnosis: Hereditary elliptocytosis, thalassaemia trait and normal Rh subtype CDe cde(R_1r).

Case S5, sister, aged 20 years, had no complaints and she had no clinical or haematological abnormality except her red cells showing elliptocytosis (fig. 1d). Haemoglobin A_2 was normal (2.06%).

Diagnosis: Hereditary elliptocytosis.

The propositus (S3) and the father (S1) had decreased fragility both of fresh and incubated blood. The sister (S4) showed some of the cells resistant both before and after incubation. The other sister (S5) and mother (S2) had normal fragility. X ray of the skull and hands were normal in all of them.

Discussion

The blood picture and the results of the haemoglobin analysis of the propositus, the father and the elder of the 2 sisters revealed that they had elliptocytosis with thalassaemia trait. Serologically S1 was found to be D^u (CDᵘe cde) and family investigations revealed S3 having a rare genotype CDᵘe CDe ($R_1^uR_1$). S1, S3 and S4 had normal alkali-resistant haemoglobin but elevated haemoglobin A_2 levels. In Ceylon, there is a preponderance of A_2-thalassaemia in families of patients with β thalassaemia major [20]. In the diagnosis of thalassaemia trait, deter-

Table 1 Haematological data

Case	Age years	Hb g%	PCV %	MCV μm^3	MCHC %	Reticulocytes %	Red cell morphology						Osmotic fragility, fresh and incubated blood
							aniso-cytes	poikilo-cytes	ellipto-cytes	spero-cytes	hypo-chromia	target cells	
S1 Father	50	11.1	39	97.5	28.5	1.2	+	+	++	0	+	++	markedly decreased
S2 Mother	40	12.4	42	95.0	29.3	0.4	0	0	0	0	+	0	normal
S3 Propositus	22	10.2	37	92.5	30.3	0.4	+	+	+++	0	0	++	markedly decreased
S4 Sister	21	9.4	35	97.2	26.9	0.4	++	+	+++	+	+	++	partly decreased
S5 Sister	20	14.4	46	93.9	31.3	0.4	0	0	++	0	0	0	normal

Table II Results of liver function and other investigations

Case	Liver function tests						Hb-A_2 fraction %†	Alkaline resistant Hb. %	Glutathione mg %‡	Haptoglobin type	Blood groups	
	bilirubin mg %	ZnSO4 turbidity	thymol turbidity	thymol flocculation	alkaline phosphatase KA units	cephalin cholesterol					ABO phenotype	Rh genotype
S1	0.4	4	-	nil	8	+2	4.22	0.875	28.5	2-1	O CcD^uee,	CD^ue cde, (R_1^ur)
S2			-	-	-	-	1.96	1.96	32.0	2-1	B CcDee	CDe/cde, (R_1r)
S3												
Propositus	0.3	12	2	nil	9	+1	3.80	1.33	30.0	0-0	O CCD^uee,	CD^ue CDe, ($R_1^uR_1$)
S4	0.6	24	3	nil	10	++½	4.02	1.76	34.0	0-0	O CcDee,	CDe/cde, (R_1r)
S5	0.4	19	2	nil	13	+2	2.06	0.84	29.0	2-2	B ccddee,	cde/cde, (rr)

† Normal range 1.9–3.1%. Sickling absent. G6PD (screening test) Normal activity.
‡ Mean value 40.0 mg %. Direct Coombs tests negative. No inclusion bodies.

mination of Hb A_2 is more reliable. According to WASI *et al* [21] in the presence of iron deficiency anaemia the relative amounts of Hb A_2 may be lowered considerably in normal people and thalassaemia trait and may be so lowered as to miss the diagnosis of thalassaemia trait. Our cases had low MCHC and this may have been complicated by iron deficiency.

S1, S3 and S4 did not have any clinical abnormality and there was no evidence of haemolysis as judged by the normal serum bilirubin and reticulocyte count though S4 was somewhat anaemic. The youngest sister (S5) was a case of inherited elliptocytosis. In the great majority of cases reported elliptocytosis had been a harmless anomaly.

The fact that 75% of the cells showed elliptocytosis cannot be explained only by the presence of β thalassaemia trait in S1. Further, the presence of elliptocytosis alone in S5 in the absence of other haematological abnormality supported the finding of elliptocytosis in the father (S1) to be truly hereditary elliptocytosis and not the one which commonly is associated with thalassaemia trait. In S4 there were microspherocytes and this has been known to occur with hereditary elliptocytosis [22]. The blood films of S1, S3 and S4 showed an increased number of target cells suggestive of thalassaemia trait in addition to the elliptocytosis. The former was confirmed by haemoglobin analysis.

In this family the gene for elliptocytosis did not travel with R_1 (CDe), R_1^w (CD^we) or r (cde) since all 3 siblings did not have either R_1^w or r but showed elliptocytosis inherited from R_1^wr (CD^we cde) father. This family therefore deviated from the usual linkage reported by several workers and analysed by MORTON [23].

Hp (2–1) X Hp (2–1) mating (S1 X S2) has produced no detectable haptoglobin bands in S3 and S4 on starch gel electrophoresis. 2 genetic conditions namely thalassaemia trait and elliptocytosis in the propositus (S3) and his father (S1) may not be responsible for the absence of Hp bands, since S1 showed a distinct (2–1) type on electrophoresis but not S3, both having elliptocytosis and thalassaemia. Again the sister (S4), a thalassaemia carrier, did not show any Hp bands. It may seem that the absence of Hp bands in S3 and S4 is independent of other genetical conditions observed in this family. It may have been brought about by further lowering of the Hp levels in their blood by some haemolytic processes. It is found in some populations of SE Asia and New Guinea that low haemolytic episodes may not enable the Hp phenotype to be assigned by usual typing using starch gel electrophoresis [24].

S1 and S3 in this study had markedly reduced fragility both of fresh and incubated cells This marked resistance could be explained by the coexistence of thalassaemia Thalassaemia itself causes increased resistance S4 however, had only a part of the cells showing decreased fragility, a greater part fell within the normal range She had microspherocytes and this could account for the greater part of the curve lying within the normal range Though the fragility is normal in uncomplicated elliptocytosis, increased resistance may be suggestive of the presence of another associated abnormality like thalassaemia or a haemoglobinopathy AVERY [25] described a case of Hb C and elliptocytosis whose osmotic fragility was normal or near normal before incubation and shifted in the direction of increased resistance on incubation

Cases have been described of elliptocytosis associated with oxycephaly [26] and defects of the lateral incisors [27] None of our cases had these defects PENFOLD and LIPSCOMB [28] described a Jewish family with hereditary elliptocytosis and hereditary haemorrhagic telangiectasia

Hereditary elliptocytosis is inherited as a Mendelian dominant and is equally common in males and females According to MORTON [23] there may be more than one gene responsible for the morphological appearances of the red cell Interesting problems arise when two or more genes for inherited haemolytic disorders co-exist in the same population and interact so that a person inheriting both mutant gene is at a disadvantage In SE Asia for example, β thalassaemia and Hb E are both present and the presence of the 2 genes in an individual leads usually to a moderately severe blood disorder In our cases the elliptocytic trait has been associated with the thalassaemia trait but the individuals were seemingly unaffected It is apparent that in these cases no interaction has occurred Similarly in the family with hereditary persistence of foetal haemoglobin and elliptocytosis no evidence of interaction was seen in individuals heterozygous for these genes [13] Elliptocytosis associated with Hb C [25] and Hb S [12] were found in a case with interaction between an inherited spherocytic gene and thalassaemia gene to the individuals disadvantage

According to the reports of association of hereditary elliptocytosis with β thalassaemia there seems to be no evidence to show *mutual enhancement* of the involved genes [8, 9 29] But in the family reported by PERILLIE and CHERNOFF [10] there is evidence of haemolytic anaemia being the result of the summation of the clinical effects of the genes *for* hereditary elliptocytosis and β thalassaemia in the same individual *In*

our cases there seems to be no evidence of any overt haemolytic anaemia in the individuals carrying the genes for elliptocytosis and β-thalassaemia. The fact that 3 of the 4 members in this family showing increased number of elliptocytes had also β-thalassaemia does not necessarily indicate that this abnormal morphology of the red cells is associated with thalassaemia. This is borne out by the fact that the other member in the family showing elliptocytosis is found to be free of β-thalassaemia trait with normal amount of Hb A_2. From the limited genetic data available there is reasonably good evidence that the genes for β-thalassaemia and hereditary elliptocytosis in one family reported here are not allelic.

Acknowledgements Our thanks are due to Dr D P. K. M DE SILVA, Pathologist, Government Hospital, Gampaha, W P, Ceylon, to Dr. L. D SANGHVI, M Sc., Ph D (Columbia), Head, Epidemiology Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 12, India, for their help, to Dr. A. J. BAXI, M Sc, Ph D, Blood Group Reference Centre, Seth G S Medical College, Parel, Bombay 12, India, for the glutathione estimation in the red cells and to the Superintendent of Health Services, Colombo, for permission to publish this paper.

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Acta haemat 46 242–253 (1971)

Parahemophilia

A Case Report

A GIROLAMI, R VENTURELLI, F RIGHINI and R SCARPA

University of Padua Medical School Institute of Semeiotica Medica'
(Director Prof M AUSTONI)
and Udine City Hospital, Blood Transfusion Center
(Director Prof R VENTURELLI)

Abstract A case of congenital factor V deficiency is presented The proposita is a 40-year old female who has a prolonged prothrombin time corrected by the addition of adsorbed plasma but not by the addition of normal serum a prolonged partial thromboplastin time and an abnormal thromboplastin generation corrected by the substitution of the patient s adsorbed plasma with adsorbed normal plasma The factor V level in the patient s plasma is 6% of normal Bleeding tendency was mild Menstruation was almost always normal No hemarthrosis ever occurred The patient s mother and one of her siblings were considered to be heterozygotes

Key Words
Coagulation defects
Factor V deficiency
Parahemophilia

Congenital factor V deficiency was first described by OWREN in 1947 [30] To date only about 50 cases have been reported in international literature The main features presented by all these patients have been properly emphasized by authors who reported the latest cases or in recent reviews [2, 6, 20, 22, 23, 29 32, 33, 35, 36, 39] The description of new cases of this disease should be welcome since they may add further information about the condition

The object of the present paper is to report a patient with parahemophilia who was followed since 1957 This is the fourth patient with parahemophilia described in Italy [6, 15]

Case Report

The patient is a 40-year old white female who was first seen by us in 1957 and has been followed up by us throughout these years (fig 1 table I) The parents of the proposita were not consanguineous but they were born in the same area They

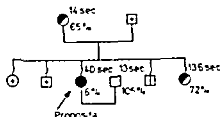


Fig. 1. Family pedigree. The numbers on the right side of each circle or square refer to the prothrombin time and to the factor V percent values. The mother and the sister of our proposita had slightly decreased factor V levels and were considered to be heterozygotes.

Table I. Behavior of clotting time, prothrombin time and partial thromboplastin time during the 12 year observation period. The minor variations are secondary to the different reagents used from time to time.

Test	Date of examination			
	Feb 1957	April 1963	Dec 1968	Nov 1970
Clotting time, min	14	18	12	16
Partial thromboplastin time, sec		–	95	106
Prothrombin time, sec	39	48	38	40

were asymptomatic. The family history was positive for bleeding tendency, since 3 maternal cousins of the proposita are considered to be bleeders. Unfortunately no coagulation study could be carried out in these 3 persons. The proposita's sister presented excessive bleeding after a tooth extraction.

The first bleeding manifestations of the proposita were easy bruising, epistaxis and occasional bleeding from the gums. At the age of 7 a tonsillectomy was not performed because of whole blood clotting time of 22 min. Menarche appeared at the age of 11 and was not particularly abundant. Menstruations have been normal or only moderately increased throughout her life.

At the age of 16 a tooth extraction was followed by profuse bleeding which required hospitalization and 1 unit of fresh whole blood. At that time the diagnosis of parahemophilia was firmly established. At the age of 26 the patient was hospitalized in preparation for multiple dental extractions. 600 ml of fresh plasma were given in 2 h and 8 tooth extractions were carried out immediately after in 1 sitting without excessive bleeding. At the end of the plasma infusion, the partial

Acta haemat 46 242-253 (1971)

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Case Report

The patient is a 40 year old white female who was first seen by us in 1957 and has been followed up by us throughout these years (fig 1 table I) The parents of the proposita were not consanguineous but they were born in the same area They

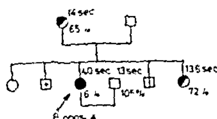


Fig. 1. Family pedigree. The numbers on the right side of each circle or square refer to the prothrombin time and to the factor V percent values. The mother and the sister of our proposita had slightly decreased factor V levels and were considered to be heterozygotes.

Table 1. Behavior of clotting time, prothrombin time and partial thromboplastin time during the 12 year observation period. The minor variations are secondary to the different reagents used from time to time.

Test	Date of examination			
	Feb 1957	Apr 1963	Dec 1968	Nov 19[illegible]
Clotting time, min	14	18	12	16
Partial thromboplastin time, sec			95	106
Prothrombin time, sec	39	48	35	40

were asymptomatic. The family history was positive for bleeding tendency since 3 maternal cousins of the proposita are considered to be bleeders. Unfortunately no coagulation study could be carried out in these 3 persons. The proposita's sister presented excessive bleeding after a tooth extraction.

The first bleeding manifestations of the proposita were easy bruising, epistaxis and [illegible] bleeding from the gums. At the age of 7 a tonsillectomy was not performed because of whole blood clotting time of [illegible] min. Menarche appeared at the age of 11 and was not particularly abundant. Menstruations have been normal with only moderately increased throughout her life.

At the age of 16 a tooth extraction was followed by profuse bleeding which required hospitalization and 1 unit of fresh whole blood. At that time the diagnosis of parahemophilia was firmly established. At the age of 26 the patient was hospitalized in preparation for multiple dental extractions. 600 ml of fresh plasma were given in [illegible] h and 8 tooth extractions were carried out immediately after it [illegible] without excessive bleeding. At the end of the plasma infusion, the partial

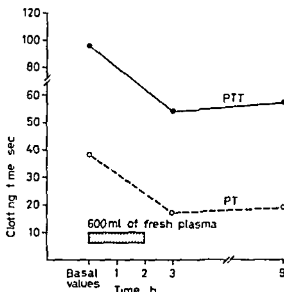


Fig 2 Almost complete correction of partial thromboplastin time (PPT) and prothrombin time (PT) after the administration of 600 ml of fresh plasma The extraction of 8 teeth carried out immediately after the transfusion was not followed by excessive bleeding No specific factor V assays could be carried out on this occasion but it is likely that a level of 20–25% of normal was reached (body weight 65 kg estimated plasma value 2 500 ml)

thromboplastin time and the prothrombin time were practically normal (fig 2) On the following day another unit of fresh plasma was given and a third unit was given on the third day No excessive oozing was noted from the sites of extraction

Altogether spontaneous bleeding manifestations have been mild During the past 3 years the patient had no important bleeding manifestation Physical examination at the time of study was negative

Material and Methods

Plastic syringes (Pharmaseal) and gauge No 19 siliconized needles 3 8% sodium citrate Calcium chloride 0 025 M Michaelis buffer solution at pH 7 3 Imidazole buffer solution at pH 7 3 Veronal buffer at pH 7 3

Tissue whole thromboplastin (Simplastin Warner Chilcott or rabbit brain thromboplastin Ortho) Activated partial thromboplastin (Cephalopastin Dade) Thrombin topical (Topostasin Roche) Thrombin (Behringwerke) Bovine fibrinogen (Armour) Charcoal filtered ox plasma (Diagen) Prothrombin free beef plasma (Hyland)

Lyophilized factor VII deficient plasma was obtained through the cooperation of Dr OWEN Department of Clinical Pathology Mayo Clinic, Rochester, Minn., USA Fresh factors VIII IX and XII deficient plasma was obtained from individuals with severe congenital deficiencies of these factors Frozen factor X deficient plasma as obtained from a patient with severe factor X deficiency

Celite a grade 1 diatomaceous earth obtained from Sigma Laboratories, was used. Ellagic acid as obtained from the K & K Laboratories Jamaica, New York The crude material was purified as previously described (13) Silicone 200 350 (Società Generale Siliconi)

Russell viper venom (Burroughs Wellcome) The dried preparation was reconstituted with the solvent supplied by the manufacturer to obtain a 1 10 000 solution Stypven-Cephalin preparation as obtained from Diagnostic Reagents Laboratories.

Adsorbed normal plasma was obtained by mixing 1 ml of citrated plasma with 0.1 ml of aluminium hydroxide suspension (Diagen) for 5 min The supernatant was then separated by centrifuging at 3,000 rpm for 5 min and the procedure repeated. The prothrombin time of the adsorbed normal plasma was always greater than 200 sec Factors II V and factors VII + X deficient substrates as supplied by the Stago Laboratories were used

Most of the results dealt within the present report refer to tests carried out in November and December 1970

Plasma was obtained by centrifuging at 5 C 1 10 citrated blood for 10 min at 2,000 rpm unless otherwise specified All tests were carried out in normal glassware unless otherwise specified The glass clotting time was performed according to the LEE and WHITE method [26] Platelets were counted according to the BRECHER and CRONKITE method [4] Clot retraction and the tourniquet test were carried out by routine accepted procedures [2, 28]. Bleeding time was determined by DUKE's method [33] The prothrombin-proconvertin test was performed according to the method of OWREN and AAS [31], modified by WARE and STRAGNELL [35] The factors II + X complex was evaluated according to a modification of the method of HICKS et al [18] Prothrombin free ox plasma was incubated with equal parts of a Russell viper venom-cephalin mixture and a 1 10 dilution of the test plasma in Michaelis buffer After a 5-min incubation period 0.1 ml of a 0.025 M $CaCl_2$ solution was added and the clotting time measured

The Stypven clotting time was obtained measuring the clotting time of a mixture of the patient's plasma and 0.1 ml of a 1 10,000 dilution of Wellcome Russell viper venom on addition of 0.1 ml of 0.025 M $CaCl_2$. The platelet rich plasma for the Stypven clotting time was obtained by centrifuging non-contacted 1 10 citrated blood at 1,500 rpm for 5 min the test was carried out in normal glassware

The Stypven-Cephalin clotting time was determined by mixing 0.5 ml of the patient's plasma with 0.1 ml of the Stypven-cephalin mixture and then by adding 0.1 ml of the usual $CaCl_2$ solution Factors II V and factors VII + X complex were determined by the methods of Owren modified by LEWIS et al [27] Factor VII activity alone was evaluated using as substrate equal parts of lyophilized factor VII deficient plasma and adsorbed normal plasma

Factor X activity was determined according to the Stypven-cephalin method Diagen charcoal filtered ox plasma was used as substrate A normal reference

Lyoph l zed factor VII def cient plasma was obtained through the cooperat on of Dr OWEN Department of Cl n cal Pathology Mayo Cl n c, Rochester M nn., USA Fresh factors VIII IX and XII def cient plasma was obta ned from ind vid uals w th severe congen tal def c enc es of these factors. Frozen factor X def cient plasma as obta ned from a pat ent with severe factor X def ciency

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Russell viper venom (Burroughs Wellcome) The dried preparat on was recon st tuted w th the solvent suppl ed by the manufacturer to obtain a 1 10,000 solu t on Stypven Cephal n preparat on as obta ned from D agnost c Reagents Labora tor es

Adsorbed normal plasma was obtained by m xing 1 ml of citrated plasma with 0.1 ml of alum n um hydrox de suspens on (D agen) for 5 m n The supernatant was then separated by centr fug ng at 3 000 rpm for 5 min and the procedure re peated The prothromb n t me of the adsorbed normal plasma was always greater than 300 sec Factors II V and factors VII + X def cient substrates as suppl ed by the Stago Labc ratories were used.

Most of the results dealt w h n the present report refer to tests carried out in November and December 1970

Plasma was obta ned by centr fug ng at 5 C 1 10 citrated blood for 10 m n at 2,000 rpm unless otherwise specif ed All tests were carried out in normal glass-ware unless otherw se specif ed The glass clott ng t me was performed accord ng to the LEE and WHITE method [26] Platelets were counted accord ng to the BRECHER and CRONKITE method [4] Clot retract on and the tourn quet test were carried out by rout ne accepted procedures [2, 28] Bleed ng t me was determ ned by DUKE s method [33] The prothromb n proconvert n test was performed a cord ng to the method of OWREN and AAS [31] mod f ed by WARE and STRAGNELL [35] The factors II + X complex was evaluated accord ng to a mod f cat on of the method of HJORT et al [18] Prothromb n free ox plasma was incuba ed with equal parts of a Russell v per venom-cephal n mixture and a 1 10 d lut on of the test plasma in M chaelis buffer After a 5 m n incubat on period 0.1 ml of a 0.025 M $CaCl_2$ solut on was added and the cl tt ng t me measured.

The Stypven cl tt ng t me was obta ned measuring the clott ng t me of a mix ture of the pat ent s plasma and 0.1 ml of a 1 10 000 d lution of Wellcome Russell v per venom on add t on of 0.1 ml of 0.025 M $CaCl_2$ The platelet r ch plasma for the Stypven cl tt ng t me was obta ned by centr fug ng non-contacted 1 10 citrated bl od at 1 800 rpm for 5 m n the test was carr ed out in normal glassware

The Stypven Cephal n cl tt ng t me was determ ned by m x ng 0.1 ml of the pat ent s plasma w h 0.1 ml of the Stypven-cephal n mixture and then by add ng 0.1 ml of the usual $CaCl_2$ solution. Factors II V and factors VII + X complex were determ ned by the methods of Owren mod fied by LEWIS et al. [27] Factor VII act v ty alone was evaluated using as substrate equal parts of lyoph l zed fa tor VII def cient plasma and adsorbed normal plasma

Factor X act v ty was determ ned accord ng to the Stypven-cephal n method [5 Dhagen charcoal f ltered ox plasma was used as substrate A normal reference

curve for factors II, V, factors VII + X complex, factors VII and X assays was constructed using serial dilutions of normal plasma in Michaelis buffer

Factors VIII and IX were determined according to the method of LANGDELL *et al* [24] modified by HARDISTY and MACPHERSON [16] The PTA level was determined according to the method proposed by HOROWITZ *et al* [21] Hageman factor activity was determined by a 1 stage method using as substrate non contacted plasma of a patient with Hageman trait [34] This latter assay was carried out in siliconized glassware

Antiproaccelerin activity was evaluated by incubating at 37° C for 2 h a mixture of patient's plasma with fresh normal plasma and a mixture of aged normal plasma with fresh normal plasma The aged normal plasma was obtained by storing pooled normal plasma at +4° C till the prothrombin time became greater than 60 sec [15]

The prothrombin consumption test was carried out on serum obtained from blood which had been allowed to stand at 37° C for 2 h [15, 33] The partial thromboplastin time was evaluated by the method of LANGDELL *et al* [24] using an activated partial thromboplastin The thromboplastin screening test was performed according to the method of HICKS and PITNEY [17] using an activated partial thromboplastin The thromboplastin generation test was carried out according to the Oxford method using as platelet substitute a non activated cephalin preparation [2] Blood fibrinogen was evaluated according to a modification of QUICK's method [12, 33]

Fibrinolysis was assayed by means of a euglobulin method [7] Thrombin time was evaluated by measuring the clotting time of a 1 2 mixture of citrated plasma and distilled water on addition of 6 NIH units of thrombin

The thromboelastogram was obtained in a Hellige 2601 D apparatus using 1 5 citrated platelet rich plasma Routine liver function tests were carried out by accepted hospital procedures.

Results

The coagulation study is summarized in table II The prothrombin time was prolonged and was corrected by the addition of adsorbed plasma and fresh normal plasma but not by the addition of aged normal plasma or normal serum The mixing of the patient plasma with equal parts of plasma of patient with factors II, VII or X deficiencies resulted in a normal prothrombin time (tables III and IV) The partial thromboplastin time was prolonged and prothrombin consumption and the thromboplastin screening test were defective The thromboplastin generation test was abnormal and was corrected by the substitution of patient plasma with adsorbed normal plasma (fig 3) Factor V resulted to be 6% of normal No factor V inhibitor was found in the proposita's plasma (table V)

Table II Coagulation study in the proposita

Test	Patient	Normal values
Platelet count	200 000	150 000–350 000
Tourniquet test	neg	neg
Bleeding time, min	3	2–5
Clot retraction	complete in 4 h	complete after 12 h
Glass clotting time, min	16	5–10
Silicone clotting time, min	43	15–35
Recalcification time, sec	535.0	100–180
Cross-recalcification time (+0.05 ml of normal plasma), sec	122.5	/
Thromboplastin screening test	26 sec in 8 min	<11 sec in 6 or 8 min
Thromboplastin generation test	30 sec in 6 min	<14 sec in 6 or 8 min
Prothrombin consumption, %	40	>90
Partial thromboplastin time, sec	106.0	<40–45
Prothrombin time, sec	40.0	13–14
Prothrombin time with Simplastin A†, sec	14.3	13–14
Prothrombin-proconvertin test, sec	26.0	23–28
Factors II + X complex, sec	22.2	21
Stypven clotting time, sec	56.0	14–20
Stypven cephalin clotting time, sec	48.0	10–13
Factor II (1-stage), %	100	80–120
Factor V, %	6	80–120
Factors VII + X complex, %	100	80–120
Factor VIII, %	95	60–130
Factor IX, %	105	60–130
Factor XI, %	60	50–130
Factor XII, %	100	50–130
Fibrinogen, mg %	530	250–500
Fibrinolysis (euglobulin), h	26	10–30
Thrombin time, sec	19	18–25
Thromboelastogram, M, mm	75	10–20
k, mm	30	6–12
ma, mm	66	50–66

† This thromboplastin contains factor V and fibrinogen

Factors II, VII, VIII, IX, X, XI, and XII were all within normal limits. Fibrinogen was slightly elevated but no significance could be attributed to this finding. There was no hyperfibrinolysis. The thrombin time was normal. The thromboelastogram showed a prolonged 'r' and 'k' but a normal 'ma'. The pattern was partially corrected by the addi-

Table III Prothrombin time correction studies

Mixture, equal parts	Time, sec
Patient s plasma	40
Patient's plasma + normal serum	58
Patient's plasma + adsorbed normal plasma	17
Patient's plasma + fresh normal plasma	15 6
Patient's plasma + aged normal plasma	38 2

Table IV Prothrombin times of mixtures of patient's plasma with the plasma of known congenital coagulation disorders

Mixture, equal parts	Prothrombin time, sec		Comment
	mixture	reference plasma	
Patient's plasma	40		
Patient's plasma + factor II deficient plasma	14 8	20 0	personal case
Patient's plasma + factor VII deficient plasma	18 5	48 0	lyophilized plasm received from Dr Owen
Patient s plasma + factor X deficient plasma	19 5	11 1	personal case
Patient's plasma + abnormal factor X (factor X Friuli) plasma	15 2	33 7	personal case
Patient's plasma + plasma of another patient with parahemophilia	37 6	34 2	personal case

tion of small amounts of ellagic acid (fig 4) Platelets and vascular tests were within normal limits

Routine liver function studies were all within normal limits The mother and the sister of our proposita had a slightly decreased factor V level and were considered to be heterozygotes The proposita's hubsand, on the contrary, was found to have perfectly normal factor V levels and was therefore considered to be normal

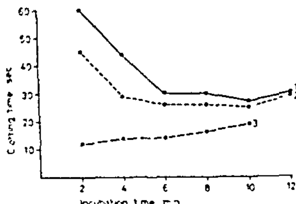


Fig. 3. Thromboplastin generation test. Curve 1 is the basal curve. Curve 2 was obtained after substitution of patient's serum with normal serum. Curve 3 was obtained after substitution of patient's absorbed plasma with absorbed normal plasma. Non-activated platelet substitute and 0.025 M $CaCl_2$ were also used in each system. Normal plasma was used as substrate in all instances.

Discussion

The main features of factor V deficiency are prolonged prothrombin time corrected by the addition of adsorbed normal plasma, prolonged partial thromboplastin time and defective thromboplastin generation [2, 10, 33]. Our patient fully meets these criteria.

The possibility that our patient had an acquired factor V deficiency may be ruled out. Liver damage can be ruled out because of normal liver function tests and of no concomitant decrease of vitamin K dependent factors. Consumption coagulopathy or fibrinolytic purpura may also be ruled out since no concomitant decrease in fibrinogen, platelets, factors II and VIII, and no hyperfibrinolysis was present.

The results of the coagulation studies are in perfect agreement with the known data on the subject. The partial correction of the thromboelastographic pattern obtained after addition of ellagic acid is not surprising. This first stage activating substance has already been shown to correct partially or completely the thromboelastographic tracing of all coagulopathies safe for Hageman trait and congenital afibrinogenemia [14]. The most striking aspect presented by our proposita concerns the mild bleeding tendency throughout her life in spite of the low factor V levels.

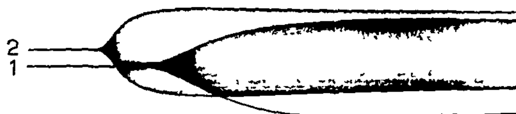


Fig 4 Thromboelastographic pattern The prolonged 'r and K are evident in tracing 1 (basal tracing) Tracing 2 was obtained after addition of 0 1 ml of 10^{-4}M ellagic acid solution to 0 9 ml of patient s platelet rich plasma The addition of ellagic acid has partially corrected the abnormality

Table I Lack of anti factor V activity in the patient s plasma on incubation at 37°C

Incubation time min	Factor V level of a 1 2 mixture of patient s plasma and fresh normal plasma %	Factor V level of a 1 2 mixture of aged normal plasma and fresh normal plasma %
0	56	52
30	58	46
60	46	46
120	40	38

The threshold for the occurrence of overt bleeding manifestations in factor V deficiency seems to be around 20% of normal [15, 36, 38] and the 6% level found in our patient was well below that level The probable factor V level obtained in our proposita after the transfusion of 600 ml of plasma was also around 20–25% of normal (fig 2) The threshold level for an abnormal TGT test in parahemophilia seems to be around 20%, too [25]

A similar striking discrepancy between factor V levels and a mild bleeding tendency was already present in the patients presented by ALEXANDER *et al* [1] and FRIEDMAN *et al* [11] There is no sure explanation for the phenomenon Therefore, it would seem that other concomitant factors may play a role in the pathogenesis of bleeding in patients

with parahemophilia. In this regard it may be interesting to note that a level as low as 5–10% of normal was maintained to be sufficient to assure adequate hemostasis for a hysterectomy [3].

Quick has suggested that bleeding manifestations in parahemophilia are similar to those observed in angiohemophilia or von Willebrand's disease [33]. This view is consistent with the bleeding that was observed in our patient.

Menorrhagia has been reported to be frequently present, but this was not the case in our patient.

The heredity pattern in our proposita seems well established. She is probably a homozygote. The mother is a heterozygote. The father was either a heterozygote himself or he was normal. In this latter case a mutation must be invoked to explain the homozygosis found in his daughter. The data available in the literature are in agreement with this interpretation [4, 23, 37].

These findings are consistent with an autosomal incompletely recessive pattern of transmission.

Several associated congenital anomalies have been described in patients with parahemophilia, namely syndactylism, epidermolysis bullosa and factor VIII deficiency [9, 10, 22]. In other patients a factor V inhibitor was demonstrated [10, 19]. None of these conditions was present in our proposita.

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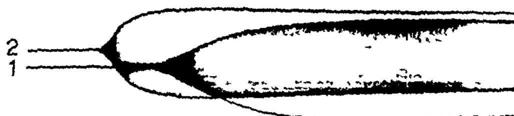


Fig 4 Thromboelastographic pattern The prolonged 'r' and 'K' are evident in tracing 1 (basal tracing) Tracing 2 was obtained after addition of 0 1 ml of 10^{-4}M ellagic acid solution to 0 9 ml of *patient s platelet rich plasma* The addition of ellagic acid has *partially* corrected the abnormality

Table 1 Lack of anti factor V activity in the patient's plasma *on incubation* at 37°C

Incubation time min	Factor V level of a 1 2 mixture of patient s plasma and fresh normal plasma, %	Factor V level of a 1 2 mixture of aged normal plasma and fresh normal plasma %
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Libri

J Caen, F Josso Y Sultan D Meyer et J P Allain L hemostase, physiologie exploration fonctionnelle Expansion Scientifique Française

Ce petit volume résume avec compétence la physiologie de l hémostase et de la coagulation sanguine ainsi que de la fibrinolyse Il traite ensuite l exploration fonctionnelle en clinique On y trouve toutes les méthodes importantes mais il y manque des indications pratiques sur les méthodes à employer pour une premiere orientation Pour le débutant il lui serait utile de se familiariser avec les méthodes simples qui permettent d exclure un dérangement de la coagulation ou de sélectionner des méthodes plus spécialisées lorsqu un résultat est pathologique Dans beaucoup d hopitaux le protocole suivant a déjà fait ses preuves temps de Quick, PTT fibrinogène activité anti thrombine nombre de plaquettes retraction du caillot temps de saignement Seuls les malades presentant un test pathologique sont examinés plus à fond par un laboratoire spécialisé La troisième partie du livre a une haute valeur didactique puisqu il présente à l étudiant les résultats obtenus dans des cas bien définis

Ce livre est à recommander aux étudiants et aux jeunes médecins s interessant a l hémostase moderne

P Miescher Genève

R G MacFarlane (ed) **The Haemostatic Mechanisms in Man and Other Animals** Symposia of the Zoological Society of London No 27 Academic Press London 1970 US $ 13 50

The symposium organized by R G MacFarlane on behalf of the Zoological Society of London constitutes a successful attempt to give a broad review of the accumulated knowledge on the haemostatic process not only in man but also in numerous animals belonging to 9 phyla The proceedings give a full report of the Symposium held in London in December 1970 A few chapters are devoted to the coagulation mechanism coagulation defects and the role of platelets in man Even though it is relatively easy to find such information elsewhere these chapters are very helpful for direct comparison The goal of the different authors has been variable from large reviews such as that presented by Needham on haemostasis in the invertebrates by Christine Hawkey on fibrinolysis in animals to very specific contributions for example on *in vivo* observations on haemostasis in the hamster by Sanders

Some attempts of a direct comparison between various species or animal classes have been made As stated by the authors such attempts are quite difficult since for the majority of species very little is known furthermore the methods used in humans or in the most commonly studied animals are not directly adaptable to other animals because of species specificity especially for comparison on a quantitative basis The chemical experimentation is in this respect more successful since the structure of physiologically equivalent proteins can be analyzed This is possible only for fibrinogen the quantitatively best represented coagulation factor This has been shown by Blombäck and also by Cartwright

The possibilities of immunological techniques have been somewhat neglected except for a presentation by Denson on abnormal clotting proteins This aspect could have been extended to the use of antisera to compare the coagulation or fibrinolysis

factors in different species, since such data exist. Nevertheless, these proceedings constitute an extremely valuable source of information and references, which can be highly recommended to anyone interested in the field of haemostasis.

F. DUCKERT, *Basel*

H.-J. KARITITZ: Hämatologischer Zytologieatlas für Praxis und Klinik. H. Marseille, München 1970. 98 pp., 434 fig., DM 30.–.

Es ist erfreulich, dass die morphologische Information in der hämatologischen Zytologie sich immer mehr von handgezeichneten zugunsten mikrophotographischer Abbildungen löst und damit zur Darstellung der Wirklichkeit übergeht. Nach dem vor 6 Jahren erschienenen Leitfaden des Referenten ist der kleine Atlas von KARITITZ das zweite Werk zum Studium der Blut- und Knochenmarkszellen im deutschsprachigen Schrifttum, das sogar ausschliesslich farbige Mikrophotogramme verwendet und noch einen sehr wertvollen Anhang über die Zytologie des Pleura-, Aszites- und Perikardpunktates enthält. Auf spezielle didaktische Gesichtspunkte scheint es der Autor weniger abgesehen zu haben, die einzelnen Knochenmarkszellen werden unter normalen und pathologischen Bedingungen nacheinander abgehandelt. Dabei ist zu begrüssen, dass stets mehrere Beispiele zum jeweiligen Thema gezeigt werden, um dem Unerfahrenen das Verständnis für die morphologische Variationsbreite hämatologischer Zellbefunde zu erleichtern. Die Abbildungen sind vorwiegend gut oder sehr gut; einige wenige Verbesserungen wären vorzuschlagen, so z. B. die Darstellung der Eosinophilen und Basophilen auf p. 35. Wünschenswert wäre auch bei einer derartigen Neuerscheinung ein Hinweis auf die wichtigsten zytochemischen Befunde bei bestimmten Krankheitsbildern (akute Leukosen, chron. myel. Leukose, sideroachrestische Anämie). Im übrigen ist aber die gesamte Thematik der hämatologischen Knochenmarkzytologie nahezu erschöpfend und sehr illustrativ behandelt, so dass das Büchlein allen an der mikroskopisch-hämatologischen Diagnostik Interessierten empfohlen werden kann.

F. HECKNER, *Einbeck*

J CAEN F JOSSO Y SULTAN D MEYER et J P ALLAIN L hémostase physiologie exploration fonctionnelle Expansion Scientifique Française

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Acta haemat. 46 257-266 (1971)

Serial Estimation of Serum, Urine, and Leukocyte Muramidase (Lysozyme) in Monocytic Leukemia

H. OHTA and H. NAGASE

Department of Medicine, Nagoya University School of Medicine, Nagoya

Abstract. Serum muramidase values were greatly increased in all patients with monocytic leukemia, moderately elevated in chronic myelocytic leukemia, and only slightly increased in acute myelocytic leukemia. Serial estimations in patients with monocytic leukemia disclosed a sharp rise in urinary muramidase levels at the initial stages of many patients, while the change in serum muramidase levels was less marked. In remission, serum muramidase returned to normal and muramidasuria disappeared. When relapse developed, serum and urinary muramidase increased to lower levels than those at the initial maximum ones. Mature monocytes were demonstrated to contain a greater amount of muramidase than those of immature monocytes.

Key Words
Leukemia
Monocytic leukemia
Muramidase

Patients with monocytic and myelomonocytic leukemia have greatly increased serum and urinary muramidase activity prior to therapy [1–5]. With respect to the origin of increased serum and urinary muramidase in patients with monocytic leukemia, a general correlation has been reported between muramidase excretion and leukocyte count [1]. Others could not obtain direct relationship between the degree of initial serum muramidase elevation and the presenting leukocyte count [3, 6].

The present study was undertaken to evaluate the significance of serial determinations of serum muramidase and daily quantitation of the enzyme in urine throughout the entire course of monocytic leukemia in the early specific diagnosis and as a therapeutic and prognostic indicator. Furthermore, serial estimation of leukocyte muramidase was performed in an attempt to relate the change in serum and urinary muramidase level to the nature of the presenting leukocytes during the course of the leukemia as well as to their leukokinetics.

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7 Der oder die Autoren sollen nicht alter als 40 Jahre sein

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10 Den Preisrichtern ist es gestattet die Arbeit auch weiteren Gutachtern zur Beurteilung vorzulegen

Sekretariat Dr K G v BOROVICZENY Lynarstrasse 12 *D 1 Berlin 20 (Spandau) (BRD)*

Erratum

In the paper by LUDWIK GROSS which appeared in the current issue of Acta Haematologica vol 45 No 4 (pp 218 231) 1971 entitled Studies on the Nature of Acquired Immunity Against Leukemia in Guinea Pigs on page 226 after the subheading Attempts to increase the resistance of the immunized animals by a second intradermal inoculation of leukemic cells (booster dose) the paragraph in small print should read as follows

«When 10 immunized females that had received a booster dose were challenged by a subcutaneous inoculation of leukemic cells none developed leukemia whereas out of 9 immunized guinea pigs that had not received a booster dose 2 developed leukemia following a similar challenge In a parallel experiment carried out on males 2 out of 7 immunized animals that had received a booster dose developed leukemia following a subcutaneous challenge as compared with 1 out of 2 immunized males challenged without a prior booster inoculation»

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The present study was undertaken to evaluate the significance of serial determinations of serum muramidase and daily quantitation of the enzyme in urine throughout the entire course of monocytic leukemia in the early specific diagnosis and as a therapeutic and prognostic indicator. Furthermore, serial estimation of leukocyte muramidase was performed in an attempt to relate the change in serum and urinary muramidase level to the nature of the presenting leukocytes during the course of the leukemia as well as to their leukokinetics.

Methods

Serum and urinary muramidase were measured before treatment in 56 patients with acute or chronic leukemia and other hematologic disorders Muramidase determination was also performed in 54 patients with sarcoidosis with lung involvement and slight to moderate monocytosis in peripheral blood Thirty five normal, healthy adults (men and women) served as control

Leukemic patients were classified as to type on the basis of bone marrow and peripheral blood morphology including May Giemsa stain, supravital stain, phase contrast microscopy, and cytochemical technique such as peroxidase, acid and alkaline phosphatase, non specific esterase, PAS and Sudan black B staining reactions [7-9]

Muramidase was measured by the lysoplate technique of OSSERMAN and LAWLOR [1] using purified human muramidase as the standard, since human muramidase has different activity from that of egg white muramidase Human muramidase was isolated from the urine of patients with monocytic leukemia with slight modification of the method of ALDERTON *et al* [10], using bentonite adsorption and elution with 5% aqueous pyridine adjusted to pH 5 0 with sulfuric acid The human muramidase thus obtained had 8 times grater activity than hen's egg white enzyme (Sigma Chemical Co) The purity of the isolated muramidase was verified by electrophores is Serum and urine samples were added into wells of agar plates containing uni formly suspended *Micrococcus lysodeikticus* and compared with purified human muramidase standard of 5, 10, 50, 100, 500 and 1,000 μg/ml applied to each plate Urinary muramidase excretion was determined on the aliquots of 24 hour urine collections.

Acid and alkaline phosphatase were assayed by the procedure of BESSEY *et al* [11] with minor modification, using disodium *p* nitrophenyl phosphate as substrate

Leukocytes isolated from whole blood [7] were assayed for enzyme activity after 5 cycles of freezing and thawing, and the enzyme activity expressed as micrograms per 10^9 cells (muramidase), and micromoles of *p* nitrophenol liberated by the enzyme from the substrate (unit) per 10^9 cells per hour (acid and alkaline phospha tase)

Results

Pretreatment serum muramidase levels The results of serum muramidase estimations at the time of diagnosis and prior to anti-leukemic therapy are given in table I The maximum value in each case was adopted when serial estimations were performed

Pretreatment urinary muramidase levels All patients with monocytic leukemia excreted large amounts of muramidase varying from 160 to 3,910 mg per day with a mean of 802 mg per day Since the degree of muramidasuria at the initial stage of the disease changed greatly with the progression of the diseased state, the maximum value among the ones observed by daily estimation in each case was taken up in table I

Table I Serum and urinary muramidase levels in patients with various hematologic disorders and sarcoidosis

Diagnosis	Number of cases	Serum (µg/ml) mean ± SD (range)	Urine (mg/day) mean (range)	Number of patients with muramidasuria
Normal	35	7.0 ± 1.3 (4.0–9.0)	0	0
Acute myelocytic leukemia	16	10.0 ± 6.3 (2.7–29.0)	0.1 (0–1.2)	2
Chronic myelocytic leukemia	7	17.5 ± 5.7 (10.5–26.0)	0.6 (0–1.9)	4
Monocytic leukemia	14	47.8 ± 17.3 (25.0–75.0)	802 (160–3 910)	14
Acute lymphocytic leukemia	6	2.9 ± 1.2 (1.2–4.6)	0	0
Chronic lymphocytic leukemia	1	4.0	0	0
Multiple myeloma	8	9.1 ± 3.5 (4.3–14.5)	0.4 (0–1.6)	2
Malignant lymphoma	4	9.0 ± 2.4 (6.0–11.0)	0	0
Sarcoidosis	54	12.0 ± 4.3 (4.0–23.0)	0.6 (0–6.8)	7

Much less urinary muramidase was also detected in some patients with acute myelocytic leukemia, chronic myelocytic leukemia, multiple myeloma, and sarcoidosis than in those with monocytic leukemia, and in the former groups successively positive findings were rare in the repeated determinations. No muramidase or only negligible amounts below 2 µg/ml were detected in unconcentrated urines of normal healthy persons, though in concentrated urine measurable quantities of the enzyme were found in this group. No patient had urinary muramidase activity when the serum level fell below 20 µg/ml but not all serum levels above the value were associated with muramidasuria.

No patient was in renal failure during the initial stages of the disease.

Serial serum and urinary muramidase estimations in monocytic leukemia. Serial determinations of serum muramidase and daily quantitation of the enzyme in urine were performed in 10 patients with monocytic leukemia. When these patients were first seen, peripheral leukocyte counts ranged from 5,300 to 125,000/mm³ with leukocyte differential count of 40 to 97% monocytes and immature cells. No direct correlation was observed between the level of serum muramidase and the number of the circulating leukemic cells. Moderate to marked anemia and thrombocyto-

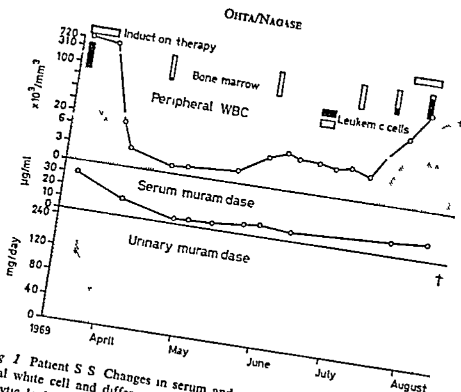


Fig 1 Patient S S Changes in serum and urinary muramidase activity as well as total white cell and differential count in the course of remitting and relapsing monocytic leukemia Shaded parts of white cell and bone marrow cells represent monocytes and blast forms in the differential count

penia were noted in all patients and blood urea nitrogen varied between 8 0 and 20 mg/100 ml

The change in the leukocyte counts and the serum and urinary muramidase levels during the course of one patient who was induced into complete remission and later developed a relapse is shown in figure 1 In accord with the change in the leukocyte count induced by chemotherapy, the serum muramidase levels decreased gradually and remained within normal range during the remission Urinary muramidase diminished more rapidly and no muramidasuria was detected in remission Serum muramidase began to increase one week after the development of the relapse Muramidase excretion in urine appeared again much later than the elevation of the enzyme in serum and, thereafter, serum and urinary muramidase levels increased gradually without reaching the initial levels

Summary of the change in serum and urinary muramidase levels in 10 serially measured patients with monocytic leukemia from the initial through the worst stage before therapy, remitted and relapsed status is

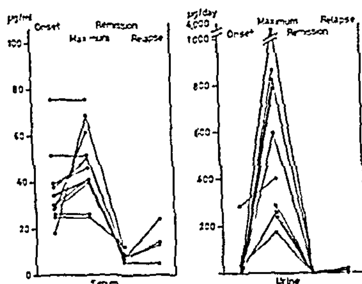


Fig. 2. Summary of the changes in serum and urinary muramidase activity determined at the initial, the worst stage before therapy, remission and relapse in each patient with monocytic leukemia.

shown in figure 2. Generally, the initial serum muramidase levels were lower than those at the worst stage, though the differences in the enzyme levels at both stages were not so marked. In all cases in which the complete remission was induced the serum muramidase levels decreased and remained in the normal range. After relapse developed, a rise (except for one case) in the serum enzyme to a lower level than at the initial stage was observed.

When chemotherapy failed to induce complete remission, serum enzyme remained elevated or above normal, and recrudescence was associated with concomitant increase in serum muramidase level.

The urinary muramidase level in the course of the disease changed remarkably. Low initial urinary enzyme values ranging from 10.6 to 33.6 mg per day, except for one case excreting 285 mg per day, were followed by a sharp rise in the activities reaching extreme high levels at the advanced stage and disappearance of urinary excretion in remission. Urinary muramidase increased if relapse developed to a much lower level than the initial highest level several days after the increase of leukocyte

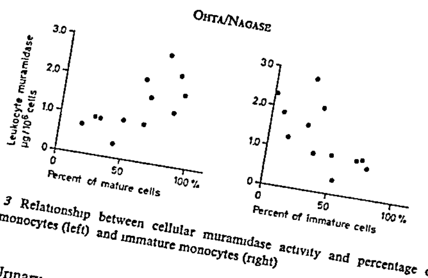


Fig 3 Relationship between cellular muramidase activity and percentage of mature monocytes (left) and immature monocytes (right)

count Urinary muramidase remained at high level in some nonremitting patients

Leukocyte muramidase activity Muramidase activity of leukocytes from patients with monocytic leukemia varied widely, possibly due to a wide range of maturity of monocytic cells and/or the diverse cellular characteristics as well as a differential cellular count in each case Figure 3 (left part) shows the relationship between leukocyte muramidase and percent of mature monocytes capable of phagocytosis Included in these leukocytes is a small number of neutrophils, since mature monocytes and neutrophils contain equivalent amounts of muramidase [12, 13] At the right part of figure 3 cellular muramidase is plotted against percent of immature monocytes with round nuclei and basophilic cytoplasm on Giemsa stain, having no phagocytic activity The correlation coefficient (r) of a linear function for cellular muramidase and percent of mature monocytes was significant at $p<0.05$, and inversely correlated were the cell muramidase and percent of immature monocytes with significance at $p<0.05$

Cellular muramidase and acid and alkaline phosphatase activities were compared between cells before therapy and cells appearing in relapse in 3 cases (table II) In 2 cases, compared to the high cellular muramidase activity at initial stage, markedly decreased cellular activity was noted in relapse, when immature cells without phagocytic capability predominated in the bone marrow and peripheral blood Similar results were obtained with respect to acid and alkaline phosphatase, though the decrement in cellular enzyme activity in relapse was less marked than that of muramidase In another case (T A), greater amounts of leukocyte muramidase and acid

Table II Comparison of leukocyte muramidase and acid and alkaline phosphatase activities measured at onset and in relapse

Patient	Muramidase, μg 10^4 cells		Acid phosphatase unit/10^4 cells		Alkaline phosphatase unit/10^4 cells	
	onset	relapse	onset	relapse	onset	relapse
S M	1.33	0.18	0.367	0.155	0.010	0.004
S S	2.08	0.45	0.584	0.250	0.006	0.003
T A	0.61	1.10	0.290	0.320	0.006	0.011

and alkaline phosphatase were demonstrated in relapse than those at the initial stage with predominating immature cells.

Discussion

The results of the present study indicate that pretreatment serum muramidase activity is greatly elevated in patients with monocytic leukemia, moderately elevated in chronic myelocytic leukemia, only slightly increased in acute myelocytic leukemia, and reduced in the lymphocytic forms of leukemia. These results are in accord with the reports of JOLLÈS et al. [14, 15] and others [1–4]. The determination of serum muramidase activity in patients with acute leukemia has proven clinically useful especially for differentiation between monocytic and myelocytic leukemia and also between myelocytic and lymphocytic leukemia, since difficulty has often been encountered with the discrimination among acute forms of leukemia based on the morphologic findings of blast cells.

Slight to moderate increase in serum muramidase level in patients with sarcoidosis [1] was confirmed in this report.

Relatively low urinary muramidase levels at the initial stage of several patients with monocytic leukemia were followed by a sharp rise in the enzyme activity reaching the extreme high level within several days (fig. 2). In one patient, with a past history of lung tuberculosis and operated stomach cancer, it was difficult to determine whether peripheral blood monocytosis might be due to exacerbation of previous disease or early stage of monocytic leukemia. Elevated serum muramidase from the onset and progressive increase in urinary muramidase were observed in this patient who later developed overt leukemia. A few patients who were first seen at an advanced stage of leukemia showed a large quantity of muramidase in

the urine These results speak for the usefulness of daily estimation of urinary muramidase for differential and early diagnosis

PERILLIE *et al* [2] and WIERNIK and SERPICK [3] reported that a sudden increase in serum muramidase level in patients in remission preceded by several days the development of overt evidence of hematologic relapse In the present study, however, serum levels did not increase before the evidence of marrow relapse and increased peripheral leukocyte count in any case Muramidasuria appeared several days after the hematological relapse, reaching lower than the initial value The results suggest that determination of serum muramidase may not be useful to know the relapse in advance in whom complete remission was achieved On the other hand, in patients in whom chemotherapy failed to induce complete remission, the serum and urinary muramidase remained elevated and varied in accord with the change in leukocyte and its differential count, serving as a good indicator for following the diseased state up and for the practical management and intensive therapy Urianry muramidase disappeared in nonremitting patients when serum muramidase levels were lowered below the renal threshold, that is about 20 $\mu g/ml$

As shown in figure 3 leukocyte muramidase activity correlated with the percent of mature monocytes with phagocytic activity and inversely correlated with immature cells, indicating that mature monocytes contain greater amounts of muramidase than immature monocytic cells These data are in agreement with the report on the granulocytic cell series of NOBLE and FUDENBERG [16] who demonstrated a parallel, in general, between the maturity of the granulocytes and their muramidase content

In remission cellular muramidase activity increased to nearly the normal level with restoring normal leukocyte differential count Interestingly enough, in some cases, much less cellular muramidase content was observed in relapse than at initial stage Together with lower values of acid and alkaline phosphatase in relapse than at initial stage, the observation may indicate that the alteration of the cell characteristics, in accordance with change in morphologic features (anaplastic transformation) occurred in relapse in some patients who had experienced complete remission The lower serum and urinary muramidase levels in relapsing patients than in patients at initial stage may be accounted for by such a change in the nature of presenting leukocytes Contrary relationship was encountered in one case at the early relapsing stage, exhibiting higher cellular enzyme activity than that at onset In nonremitting patients leukocyte muramidase levels did not change markedly

Earlier reports showing a close relationship between monocyte count and serum muramidase level suggested that monocyte series may be a main source of the elevated serum muramidase in monocytic leukemia. Since no marked elevation of serum and urinary muramidase was found in patients with chronic myelocytic leukemia having equivalent leukocyte muramidase to those with monocytic leukemia, it may be necessary to postulate other mechanisms than only the release of the enzyme from circulating leukocytes for increased muramidase quantity in monocytic leukemia. Capability of exudation of mononuclear cells in patients with monocytic leukemia followed by transformation into macrophages in tissues with marked increase in muramidase activity [17, 18] may contribute to the production of large quantities of muramidase in those patients.

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Acta haemat. 45: 267–270 (1971)

Serum Immunoglobulins in β-Thalassaemia After Splenectomy

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Abstract. Serum immunoglobulin (IgG, IgA, IgM) levels have been determined in 22 splenectomized and 50 nonsplenectomized patients with β thalassaemia major. Compared with 29 control subjects, all 3 immunoglobulins were statistically increased in the two thalassaemic groups. Splenectomized patients had higher levels of IgG and IgA, while IgM levels were found almost the same in both groups.

Key Words
Immunoglobulins
Splenectomy
Thalassaemia

Increased incidence of severe infection following splenectomy in patients with β thalassaemia has been reported [4, 6, 7, 9] and as one of the functions of the spleen is to contribute to antibody production, the question which arises is, whether after splenectomy there is a diminished antibody synthesis in these patients.

CAROLINE *et al.* [2] measuring serum immunoglobulin levels in 7 splenectomized patients with thalassaemia major, found increased values for all 3 (IgG, IgA, IgM) immunoglobulins while SCHUMACHER [8] in a group of 9 patients found only IgG increased.

The purpose of the present investigation was to extend these studies in a larger number of patients and to compare the values with immunoglobulin levels from nonsplenectomized thalassaemic subjects.

Material and Methods

Serum immunoglobulin concentrations (IgG, IgA, IgM) were determined by radial immunodiffusion technique using Immunoplates (Hyland Laboratories, Los Angeles, Ca.) in 22 patients splenectomized for hypersplenism associated with β thalassaemia major. The results were compared with serum immunoglobulin lev-

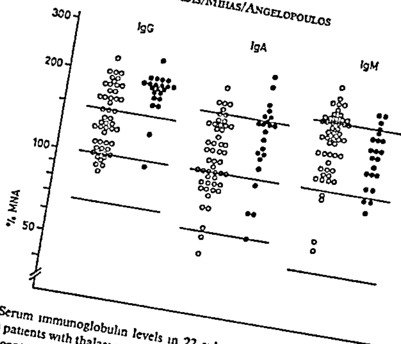


Fig 1 Serum immunoglobulin levels in 22 splenectomized (●) and 50 nonsplenectomized (○) patients with thalassaemia major shown as percent MNA against 2 SD ranges found in 29 controls

els in 50 nonsplenectomized thalassaemic patients and 29 normal blood donors. The ages of the patients ranged from 5 to 28 years and the splenectomy had been performed 1–8 years previously

The serum immunoglobulins were measured against the manufacturer's standards as stated in mg/100 ml Since normal levels show a log normal distribution all the statistical analyses were done using log normal analysis as recommended by Hobbs [5] Because of the variability of absolute standards in mg/100 ml the results were calculated as percentage of our own mean normal adult (MNA) as found in blood donors. These together with their 2 SD limits were used as the log normal ranges in figure 1 Therein the two thalassaemic groups are plotted so their increases relative to normal can be easily visualized

Results

The results are given in mg/100 ml in table I and shown as percent MNA in figure 1 It can be seen that levels of all 3 immunoglobulins are statistically increased ($p<0.001$) in patients with β thalassaemia compared with normal controls Also splenectomized patients have higher levels of IgG and IgA than nonsplenectomized while for IgM there is no statistically significant difference in the two groups

Table I. Log-normal analysis of serum immunoglobulins in 29 normal adults (controls) and 22 splenectomized and 50 nonsplenectomized patients with *β*-thalassaemia major

	IgG, mg/100 ml			IgA, mg/100 ml			IgM, mg/100 ml		
	−2 SD	mean	+2 SD	−2 SD	mean	+2 SD	−2 SD	mean	+2 SD
Controls	773	1 141	1,684	156	256	418	47	91	170
Splenectomized	1 420	2,062	2,995	186	335	601	77	130	218
Nonsplenectomized	914	1 604	2,746	169	283	473	74	138	258

Discussion

The presented data confirms the findings of previous workers. Apparently, there is no evidence of antibody deficiency which can explain the increased incidence of infections after splenectomy in thalassaemic patients. Levels of all 3 immunoglobulins were increased compared with normal controls, and there was a further increase for the IgG and IgA after splenectomy. There was no further increase for the IgM but it remained higher than in normal subjects. It seems that the factors resulting in the further increase of IgG and IgA do not operate for the IgM after the removal of the spleen. This may be because the spleen is a major source of the earliest appearing antibody, usually of the IgM class [3, 11].

Liver dysfunction and hyperplasia in the reticulo-endothelial system are the explanations offered for increased γ-globulin levels in patients with thalassaemia major [1, 10]. The further increase of the IgG and IgA possibly represents the loss of balance, with overcompensation of the remaining part of the RES, after the removal of the spleen. Alternatively, it could be the compensation of the humoral immune mechanisms, after the loss of an important part of the blood-clearing system of cellular immunity.

Acknowledgement. This investigation was supported by A. Frangistas research grant.

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Acta haemat. 46 271-274 (1971)

The Effects of Mustine Hydrochloride on the Colony Forming Units of Murine Bone Marrow[1]

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Abstract. The sequence of changes in the CFU content of murine bone marrow has been studied during the brief period of survival which follows the i.v. administration of a single lethal dose (100 µg) of mustine hydrochloride. Within 24 h a tenfold decrease in the number of CFU which can be aspirated from a single femoral diaphysis has been observed. This is followed by an increase which restores the CFU content of the femoral diaphysis to control values between the 4th and the 8th days after treatment.

Key Words
Colony forming units
Mouse bone marrow
Nitrogen mustard

BRUCE *et al.* [1] reported a profound decrease in the number of colony-forming units (CFU) in murine bone marrow 1 day after treatment with mustine hydrochloride (HN_2, nitrogen mustard) and described an exponential decrease in the fraction of surviving CFU as the dose of mustine hydrochloride was increased. The occurrence of a phase of regeneration which restores the cellularity of the bone marrow within 8 days of the administration of a lethal dose of mustine hydrochloride [2] suggests, however, that on the 4th day the hypocellular bone marrow of the mouse must contain an appreciable number of stem cells.

The repopulating ability of rat bone marrow certainly approximates to normal values 4 days after the administration of a dose of nitrogen mustard (HN_2) which effects a tenfold decrease in its nucleated cell content. But this tenfold decrease in nucleated cell content, which is maxi-

[1] This investigation was supported by a grant from the Medical Research Council.

[2] In receipt of a Scholarship for Training in Research Methods awarded by the Medical Research Council.

mal on day 4, is accompanied by only a twofold decrease in repopulating ability, which is maximal on day 1 [3].

The present investigation has been undertaken in an attempt to define the sequence of changes in the CFU content of murine bone marrow following the i.v. injection of a lethal dose of mustine hydrochloride.

Materials and Methods

Specific pathogen-free female albino mice (CS1/ASH), weighing between 20 and 25 g have been used throughout this investigation. These mice were housed under clean conditions in laminar air-flow cabinets and provided with clean food and boiled acidified water in unrestricted quantities.

Bone marrow donors each received an i.v. injection of 100 μg mustine hydrochloride (Boots Pure Drug Co., Nottingham) and were killed by exposure to ether vapour after 1, 4 or 8 days. Suspensions were prepared from the contents of the medullary cavities of the cleaned femoral diaphyses, which were aspirated gently into measured volumes of Eagle's medium.

The cell content of these suspensions was determined using a model B Coulter counter and checked using routine haemocytometric techniques. Cell suspensions were diluted to provide the various cell doses required for injection in 0.5 ml Eagle's medium. Cells were injected i.v. into groups of at least 10 recipients which had been exposed to a lethal dose of whole body x-irradiation an hour or so previously (820 rad, 300 kVp, 5 mA, 5.0 mm aluminium + 0.5 mm copper filtration, dose rate 60 rad/min).

Bone marrow recipients were autopsied after 10 days. Their spleens were removed, fixed for 24 h in aqueous Bouin's fluid and transferred to 70% alcohol for storage. The number of discrete colonies per recipient spleen [4] was used to estimate the CFU content of the donor femoral diaphyses.

Results

The sequence of changes in the nucleated cell content and in the CFU content of the bone marrow aspirated from the femoral diaphysis (table I) is illustrated in figure 1. The number of CFU is decreased to 10% of the control value 1 day after the administration of 100 μg mustine hydrochloride but a rapid increase ensues and the number of CFU/femoral diaphysis is restored to values within the normal range between the 4th and the 8th days after treatment. The nucleated cell content which is maximally depressed on day 4 is restored to values approaching the normal range by the 8th day.

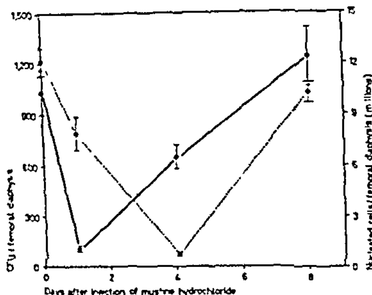


Fig. 1. Nucleated cells and colony forming units (CFU) in the medullary cavity of the femoral diaphysis after the i.v. injection of 100 µg mustine hydrochloride. ●—● CFU ● - - ● Nucleated cells.

Table 1. Nucleated cells and colony forming units (CFU) in the medullary cavity of the femoral diaphysis after the i.v. injection of 100 µg mustine hydrochloride

Days after injection	Nucleated cells/femoral diaphysis × 10⁶ (± SE)	CFU/femoral diaphysis (± SE)	Number of mice
0	12.1 ± 0.8	1,030 ± 130	45
1	7.9 ± 1.0	100 ± 20	50
4	0.7 ± 0.1	650 ± 70	40
8	10.3 ± 0.6	1,240 ± 170	40

Discussion

The decrease in the number of CFU which can be demonstrated in the bone marrow aspirated from a femoral diaphysis 1 day after the administration of an i.v. injection of 100 µg mustine hydrochloride to 10% of the number in controls is in good agreement with the decrease reported by Bruce et al. [1] following administration of the same dose

The increase in the CFU content of murine bone marrow which results in the restoration of values approaching the normal range by the 4th day resembles the pattern of recovery described for rat bone marrow by HARRISS and APONTE [3]

The effect of the dose of mustine hydrochloride which has been used in the present investigation upon the nucleated cell content of murine bone marrow is similar to the effect of the dose of nitrogen mustard used by HARRISS and APONTE [3] on the nucleated cell content of rat bone marrow In contrast, however, the maximum decrease in the stem cell content of murine bone marrow suggested by our use of the CFU assay is *five* times the maximum decrease in the stem cell content of rat bone marrow suggested by their use of 'repopulating ability' The reasons for this discrepancy are not clear It may reflect the use of different methods to assess changes in the stem cell content of the bone marrow, or it may be due either to a species difference or to a difference in the mode of action of the HN_2 and HN_3 derivatives of nitrogen mustard

Further work will be required to determine whether restoration of the CFU content of the bone marrow results from repair of damage to existing CFU or production of new CFU, or both

Acknowledgements It is a pleasure to acknowledge the help of Dr A C RICHES, Mrs V LITTLEWOOD Miss M HOWDLE, who prepared the illustration, and Mrs S BATTEN who prepared the manuscript

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es above 0.30 ml were not followed by any further increase in the spleen weight and in some cases caused a lower response.

Results

The effects of PHA on the bone marrow and spleen erythropoiesis and on their responses to EP as judged by their ^{59}Fe uptake capacity are shown in table I. A slight decrease in ^{59}Fe uptake by the marrow followed administration of the extract when it was given as the only treatment to either protein fasted or polycythemic mice. Response to EP given at different times after injection of PHA was also significantly lower than when PHA was omitted. Bone marrow changes of splenectomized groups followed a similar pattern either on the effects of PHA when it was given alone or in response to EP administered to mice pretreated with PHA. On the other hand administration of PHA alone caused in

Table I. Effects of PHA on the splenic and medullary erythropoiesis of protein fasted and polycythemic mice and on their response to EP

	Percent ^{59}Fe incorporation			
	protein fasted		polycythemic	
	bone marrow	spleen	bone marrow	spleen
Saline solution	8.91 ± 0.97[4]	0.23 ± 0.05	9.99 ± 0.86	0.44 ± 0.66
PHA (24 h)[1]	7.60 ± 0.84	0.36 ± 0.09		
PHA (48 h)	7.40 ± 0.71	0.41 ± 0.10	9.41 ± 2.96	0.89 ± 0.84
PHA (72 h)	8.36 ± 0.80	0.40 ± 0.09	9.21 ± 2.96	0.89 ± 0.72
PHA (96 h)	6.41 ± 0.61	0.36 ± 0.09		
PHA (24 h) + EP[2]	26.40 ± 3.17	0.87 ± 0.14	18.67 ± 2.96	6.20 ± 2.31
PHA (48 h) + EP	29.50 ± 3.70	5.87 ± 0.16		
PHA (72 h) + EP	*22.34 ± 3.09*	*6.46 ± 0.61*	20.98 ± 3.16	*7.70 ± 1.01*
PHA (96 h) + EP	*24.64 ± 2.94*	3.31 ± 0.90		
EP[3]	37.80 ± 3.13	1.70 ± 1.02	26.70 ± 3.02	3.40 ± 0.60

[1] PHA given as only treatment. Time elapsed between PHA injection and ^{59}Fe uptake measurement.

[2] PHA given prior EP. Time elapsed between PHA and EP.

[3] EP given as only treatment according schedule in Material and Methods.

[4] *Mean ± SE for groups of 8 to 10 mice, italic numbers differ from values of the group treated with EP alone by P of 0.05 or less.*

erythroid precursors in the marrow The subject of the present investigation represents an attempt to answer this question and concerns the study of effects of PHA on the number of erythropoietic precursors cells in the bone marrow For this purpose we have used the measurement of the bone marrow erythropoietic response to EP and the number of pluripotential stem cells as measured by the CFU technique

Materials and Methods

Adult C3H female mice 7 to 9 weeks of age were subjected to a protein free diet on day 0 [6] The erythropoietic response of the spleen and the bone marrow to 0.5 unit of EP given subcutaneously on days 6 and 7 was measured on day 8 by the 3 hour uptake of a 0 25 μCi dose of ^{59}Fe given intravenously and calculated as percent of the injected radioiron as previously described [19] A second group of mice were injected with PHA intravenously at -1, 2 -3 and -4 days prior to EP injection Using the same schedule for PHA administration EP was omitted in another group of mice Controls consisted of mice on a protein free diet injected on days 6 and 7 with 0 30 ml of saline The same protocol was used in another series of experiments in which protein deprivation was replaced by polycythemia produced by transfusion of 0 80 ml of washed erythrocytes given on days 0 and 1 Group of mice with EP and/or PHA and control were run as described above With the same experimental protocol the marrow response was studied in groups of splenectomized mice on a protein free diet or with induced polycythemia.

The effect of PHA on the number of CFU in the bone marrow and spleen was measured by the transplant method Prospective cell donors were started on a protein depleted diet on day 0 PHA was given to groups of animals at various times so that at the time of sacrifice all donors were on the 7th day of fasting but at 1 2 3 or 4 days from the time of PH injection Cell suspension were made as described by PORTEOUS and LAJTHA [15] and the cell concentration adjusted so that 10^5 bone marrow cells or 10^6 spleen cells were contained in 0 05 ml and these cell doses were used in all cases An aplastic hemopoietic condition in the recipients was induced by the administration of 350 ml/kg of Cyclophosphamide (Endoxan ASTA) and 50 ml/kg of Busulfan (Myleran Borroughs Wellcome & Co) as described by SANTOS and HAGHSHENASS [18] Eight days after transplantation 0.5 μCi of ^{59}Fe were injected intravenously and 4 h later the spleen was removed weighed and fixed in Carnoy's solution The radioactivity found in the spleen was calculated as percent of injected ^{59}Fe The number of colonies on the surface of the spleen were counted as described by TILL and MCCULLOCH [22] Erythropoietin was a LD fraction obtained from the filtrate of anemic rabbit plasma [14] and its potency tested against a sample of the International Reference Preparation [3] PHA was an extract of *Phaseolus vulgaris* prepared according to the technique described by RAZAVI [16] In pilot experiments it was found that spleen weight increases proportionally to the increase in the dose range from 0 05 to 0 30 ml Dos

Tables II and III show the effects of PHA on the number of CFU in the bone marrow and spleen as judged by spleen weight, number of colonies and ^{59}Fe uptake capacity of the spleen 8 days after cell inoculation A highly significant increase of the transplantation potential was found when spleen cells were obtained from donors pretreated with PHA 48–96 h earlier This is the same period in which splenic response to EP becomes maximal In contrast the cloning efficiency of bone marrow cells shows only a moderate but statistically insignificant increase

Discussion

Administration of PHA to protein fasted or polycythemic mice is followed by a number of morphologic changes in the spleen cells which are similar to those that follow injection of PHA to normal mice Twenty four hours after intravenous injection of absorbed PHA an increase in spleen weight is initiated which reaches a peak on day 3 and returns to normal values about day 6 to 7 [8 20] This increase in size of spleen is accompanied by an increase in the absolute number of cells in the organ and by remarkable changes in the lymphocytes which leads to the appearance of a large number of immature lymphocytes and blast like cells reaching a maximum percentage on day 3 A slight increase in erythropoiesis in the spleen in this period sharply contrasts with a marked tendency of marrow erythropoiesis to fall Administration of EP after PHA treatment makes this opposite behavior even more apparent. The extent and time course of changes in response to EP in the spleen closely correlated with changes in the pluripotential cells as measured by the colony forming technique The transplantation potential of the bone marrow cells instead appears to be little affected by PHA treatment

The effects of PHA on the spleen erythropoiesis of normal mice have been interpreted as the result of and increased proliferation of differentiated erythroid elements due to a nonspecific mitogenic action of PHA [8] If this interpretation were valid then in the protein-starved or polycythemic mice one would expect a greater effect on the bone marrow since under those experimental conditions normoblasts are practically absent in the spleen while a sizable normoblastic population remains in the marrow An increase in the stem cells concentration as a local action of PHA on splenic cells appears thus the most plausible explanation for the increase of both erythropoietin response and cloning efficiency of

Table II. Effects of PHA on the transplantation potential of spleen cells

Donors treated	Number of recipients	Spleen hemopoiesis[1]		
		colonies per spleen[4]	spleen weight, mg[4]	percent ^{59}Fe uptake
None	19	13.7±1.4	82.0±2.9	1.98±0.90
PHA (24 h)[2]	12	11.6±1.6	76.2±2.5	1.90±1.20
PHA (48 h)	13	*29.0±2.5*	*98.8±2.1*	*5.75±1.11*
PHA (72 h)	14	>30 (confluent)	*104.8±3.3*	*5.10±0.84*
PHA (96 h)	11	28.0±3.0	88.0±2.2	3.22±1.01
	22 (controls)[3]	1.4±0.3	34.2±0.99	0.15±0.05

[1] Hemopoiesis in the spleen on day 7 after transplantation.
[2] Hours elapsed between PHA administration to the donors and cell suspension preparation.
[3] Mice in this group received CY-BS treatment only.
[4] Mean and ± SE; italic numbers differ from those in the group that received cells from normal untreated donors by P of 0.05 or less.

Table III. Effects of PHA on the transplantation potential of bone marrow cells

Donors treated	Number of recipients	Spleen hematopoiesis[1]		
		colonies per spleen[4]	spleen weight, mg[4]	percent ^{59}Fe uptake
None	18	12.7±1.3	80±2.9	2.9±1.2
PHA (24 h)[2]	10	8.7±1.6	69±3.8	1.8±0.9
PHA (48 h)	9	14.0±3.1	79±3.0	1.8±0.9
PHA (72 h)	11	11.1±2.9	86±4.0	2.7±1.0
PHA (96 h)	12	12.7±3.2	74±3.6	1.8±1.1
	17 (controls)[3]	1.0±0.3	33±1.9	0.1±0.03

[1] Hemopoiesis in the spleen 7 days after transplantation.
[2] Hours elapsed between PHA administration and preparation of cell suspensions.
[3] Mice in this group received CY-BS treatment only.
[4] Mean ± SE.

the splenic area a moderate increase of the spleen ^{59}Fe uptake. Response to EP measured by the same parameter was strikingly higher when EP was given within the 48–96-hour period that follows PHA treatment.

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transplanted spleen cells found in these studies. A relationship between these changes and the course of blast transformation is suggested by the remarkable parallelism between both processes. Although one can only speculate on the nature of such relationship, it is clear enough to support the hypothesis that splenic lymphocytes undergoing blast-cell transformation might be directed by an adequate differentiating stimulation into other hemopoietic cell lines. Such capacity would be reversible and would recede with the rest of the morphologic and functional changes conditioned by PHA stimulation. This interpretation would imply that marrow 'lymphocytes' about which evidence has accrued to indicate that they can function as hemopoietic stem cells [1, 5, 12, 23] do not react to PHA in the same fashion as splenic lymphocytes do, reflecting thus, a true difference in their ability to give rise to hemopoietic competent progenitors in response to nonspecific stimulation. In this respect it should be mentioned that marrow lymphocytes differ from peripheral and splenic lymphocytes with respect to many other functional properties [4, 13]. A dissimilar behavior of the bone marrow and splenic erythropoiesis in response to a variety of nonspecific agents has also been reported [7, 19]. However, it should be kept in mind that since PHA acts upon a wide variety of cell lines, the increased concentration of stem cells in the spleen could be due to the increase in the number of progenitor cells of a line other than lymphocytic.

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Acta haemat 46 282–293 (1971)

Untersuchungen zur wachstumsfördernden Wirkung von Antiproteasen auf Lymphozyten *in vitro*[1]

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Abstract The influence of esterases and antiproteases on lymphocytes cultures has been investigated Phytohaemagglutinin (PHA) and certain PHA fractions exhibited antiprotease activity Some plant and animal antiproteases showed growth promoting activity in stimulated lymphocyte cultures an effect which could be reduced considerably by incubation with trypsin Cell homogenates and cell free supernatants from stimulated lymphocyte cultures contained esterase activity There is some evidence that these esterases are bound to antiproteases, a process which might be necessary for optimal lymphocyte growth

Key Words
Antiproteasen
Esterasen
Lymphozytenkultur
Phytohämagglutinin

Trotz zahlreicher Versuche, für Zellkulturen ausschliesslich künstliche Nährmedien zu verwenden, sind für ein optimales Wachstum bis heute Serumzusätze für Langzeitkulturen und Lymphozytenkulturen notwendig Es konnte von PUCK *et al* [9] die wachstumsfördernde Komponente Fetuin aus fötalem Kälberserum isoliert werden Fetuin besteht aus 2 Proteinen, die dem menschlichen α_2-Makroglobulin und α_1-Antitrypsin entsprechen Beides sind Antiproteasen, die die proteolytische Aktivität von Trypsin und anderen proteolytischen Enzymen inaktivieren Von LANDUREAU [7] und WALLIS [13] wurde vor kurzem die Ansicht vertreten, dass die wachstumsfördernde Wirkung dieser Proteine auf die Inaktivierung von während des Wachstums freigesetzten lyosomalen Esterasen zurückzuführen ist, die auf Zellkulturen zytotoxisch wirken sollen Es konnte bereits der wachstumsfördernde Effekt bestimmter Serumproteinfraktionen, die eine antiproteolytische Aktivität enthalten, auf Lymphozytenkulturen gezeigt werden [6] Es sollen im folgenden die Abhängigkeit der wachstumsfördernden Wirkung

[1] Mit Unterstützung der Deutschen Forschungsgemeinschaft

dieser Serumproteine von ihrer Antiproteaseaktivität sowie der Einfluss anderer Antiproteasen auf das lymphozytäre Zellwachstum gezeigt werden

Material und Methodik

Periphere Blutlymphozyten und AB-Serum wurden von gesunden Spendern gewonnen. Die folgenden Trypsin- und Antitrypsin-Präparate wurden verwendet: *Trypsin*: Trypure [illegible] (SBI), I S (Sigma), II S (Sigma), S-hu [illegible] ma), *Ovo-* [illegible] *ylol*® (Bayer, Leverkusen). Als Phythämagglutinin (PHA) wurde Phythämagglutinin P (Difco) und als Tuberkulin ein gereinigtes GT Tuberkulin der Behringwerke Marburg, verwandt

Esterasemessungen

Esteraseaktivität wurde gegen die Substrate *Benzoyl-Arginin-Äthyl-Ester* (BAEE), Extinktionsänderung bei 254 nm, Substratkonzentration 0,001 M, Tris-Hydroxymethyl-Aminomethan-HCl-Puffer, pH 8,5, 37°C, D = 1 cm [12], *Benzoyl-Tyrosin-Äthyl-Ester* (BTEE), Extinktionsänderung bei 256 nm, Substratkonzentration 0,001 M, Tris-Hydroxy-methyl-Aminomethan-HCl-Puffer mit Methanol und $CaCl_2$, pH 7,0, 25°C, D = 1 cm [12] oder *Benzoyl-Arginin-p-Nitro-Anilid* (BAPNA), Extinktionsänderung bei 405 nm, Substratkonzentration 1 mg/3 ml, Triäthanolamin-HCl-Puffer, pH 7,8, 25°C, D = 1 cm [1] ge [illegible] damit des Substratumsatzes gegenüber dem Trypsin-Vergleichsansatz bestimmt. 1 mIE entspricht der Hemmung von 1 µg Trypsin

PHA-Fraktionierung

Die Auftrennung von PHA erfolgte nach WEBER [14] über SE-Sephadex C-50 im diskontinuierlichen pH-Gradienten in 3 Fraktionen

Fraktion A = 1/15 M KH_2PO_4, pH 4,5 (Ausgangspuffer),
Fraktion B = 1/15 M KH_2PO_4 + 1/15 M Na_2HPO_4, pH 6,0
Fraktion C = 1/15 M KH_2PO_4 + 1/15 M Na_2HPO_4, pH 8,0

Eine weitere Fraktionierung von PHA wurde nach GOLDBERG [3] durch Perchlorsäurefällung und modifizierte Sevag-Methode durchgeführt (PHA-G).

Autoradiographie

Es wurden Lymphozytenkulturen (15 × 10⁶ Lymphozyten in 20 ml, mit und ohne PHA) für 3 Tage in Medium ohne L-Serin, L-Isoleucin und L-Lysin mit 15% AB-Serum in Gegenwart der ^{14}C-markierten Aminosäuren L-Serin (1,7 µCi, spezifische Aktivität 250 mCi/mM), L-Isoleucin (1,7 µCi, spezifische Aktivität 247 mCi/mM) und L-Lysin (1,7 µCi, spezifische Aktivität 120 mCi/mM) inkubiert. Anschliessend wurden die gewaschenen Zellen durch Frieren und Tauen zerstört. Die eingeengten Lymphozytenextrakte wurden in Anwesenheit von Trägerserum immunelektrophoretisch aufgetrennt.

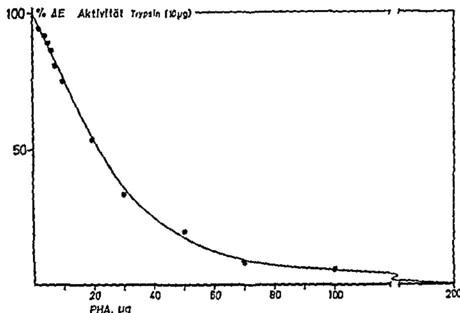


Abb 1. Hemmung der esterolytischen Aktivität von 10 µg Trypsin gegen BAPNA durch steigende Mengen von PHA (ΔE/min von 10 µg Trypsin = 100%)

Um die Menge des markierten α_2 Makroglobulins semiquantitativ zu erfassen, wurde eine radiale Immundiffusion nach OUCHTERLONY durchgeführt Hierzu wurden abgestufte Verdünnungen (im Uhrzeigersinn 1 1, 1 1,2, 1 1,5, 1·1,8, 1 5, 1:10, 1 15 und 1:20) eines Lymphozytenhomogenates aus 15×10^6 Lymphozyten in 0,5 ml mit einer konstanten Menge Trägerserum versetzt Gegen diese Mischung diffundierte ein monospezifisches Anti-α_2-Makroglobulin vom Kaninchen Nach ausgiebigem Waschen und nach Trocknung wurden Autoradiographien (Exposition 3 Wochen) durchgeführt Als Kontrollen dienten Extrakte ohne Trägerserum und Kulturen, denen erst nach Gewinnung des Zellsedimentes ^{14}C-Aminosäuren zugesetzt wurden

Lymphozytenkulturen [6]

Periphere Blutlymphozyten wurden mit Hilfe einer Nylonfasersäule isoliert Für jeden Einzelwert wurden Dreifachkulturen angesetzt Jede Kultur enthielt 3×10^6 Lymphozyten in 4 ml *Medium 199 mit 15% AB-Serum und Penicillin/Streptomycin* Einige Kulturen wurden ohne Serum angesetzt PHA, Tuberkulin oder Antitrypsin wurden zu Beginn der Kulturen zugegeben. Die Inkubation erfolgte in konischen Zentrifugenröhrchen in einem Winkel von 20° bei 37°C im CO_2 begasten Brutschrank Nach 60 Stunden wurden die Kulturen mit *4 µCi^{3}H-Thymidin oder 4 µCi^{3}H-Cytidin* (spezifische Aktivität 2,4 *Ci/mM*) für zwei Stunden beimpft und nach Waschen und Fixation des Zellsedimentes die makromolekulare RNS oder DNS mit Hilfe einer Perchlorsäureextraktion [4] gewonnen Es wurde entweder die Aktivität der Extrakte in cpm angegeben oder es wurde die spezifische Aktivität (cpm/1µg RNS bzw cpm/1µg DNS) errechnet

Ergebnisse

Die Antitrypsinaktivität von PHA ist erkennbar an der Hemmung der estereolytischen Aktivität von Trypsin gegenüber dem synthetischen Ester BAPNA Mit steigender PHA-Konzentration kommt es zu einer Zunahme der Hemmung der Trypsinaktivität (Abb 1) Die Antitrypsinaktivität des PHA fand sich in der ersten (PHA-Fraktion A, pH 4,5) und zweiten (PHA-Fraktion B, pH 6,0) Fraktion einer Chromatographie von PHA über SE-Sephadex C-50 im diskontinuierlichen pH Gradienten nach WEBER [14] und in dem nach GOLDBERG [3] durch Perchlorsäurefällung deproteinisierten PHA (Tab I) PHA-Fraktion B und PHA G wiesen neben der Antiproteaseaktivität eine deutliche lymphozytenstimulierende Fähigkeit auf

Um die Bedeutung der Antiproteaseaktivität von PHA für seine stimulierende Wirkung auf Lymphozyten zu untersuchen, wurden Mischungen

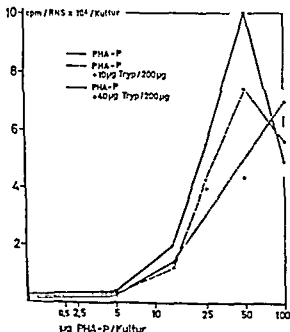


Abb 2 Wirkung von Trypsin auf die lymphozytenstimulierende Fähigkeit von PHA Veränderung der Dosiskurve von PHA durch vorherige Inkubation mit 2 Konzentrationen Trypsin

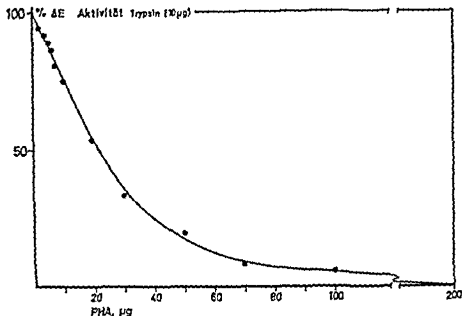


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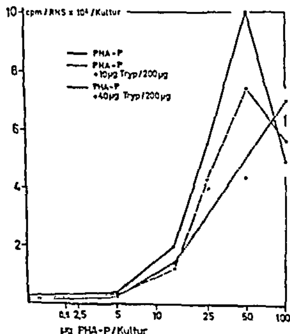


Abb 2 Wirkung von Trypsin auf die lymphozytenstimulierende Fähigkeit von PHA Veränderung der Dosiskurve von PHA durch vorherige Inkubation mit 2 Konzentrationen Trypsin

Tabelle I Antitrypsinaktivität und lymphozytenstimulierende Wirkung von PHA Fraktionen Fraktion A, B, C durch Chromatographie über SE Sephadex C-50, Fraktion G durch Deproteinisierung nach GOLDBERG (s Methodik)

PHA Fraktion	Antitrypsin aktivität mIE/µg	Lymphozytenstimulierung Dosismaximum (µg/ml Kultur)	Lymphozytenstimulierung cpm/µg RNS $\bar{x} \pm \sigma$ (n=9)	Serum präzipitation
A	0,33	o	o	o
B	0,21	25	1830±258	o
C	o	12 5	5270±670	+
G	0,33	25	3530±302	+

Tabelle II Wirkung von Proteaseinhibitoren auf unstimulierte Lymphozyten Alle Inhibitoren wurden in einer Menge von 125 mIE eingesetzt

	Un stimuliert	PHA	SBI (I S)	SBI (II S)	SBI (Sch)	Ovo-mucoid (S)	Pankreas inhibitor	LBI
cpm/DNS	310,4	14107,3	182 9	107,0	125,6	244,7	121,0	135,7
OD	0,112	0 104	0,117	0,096	0,076	0,074	0 068	0 089

aus PHA und Trypsin verwandt Hierzu wurde PHA mit 2 Konzentrationen Trypsin inkubiert (30 min, 20°C) und die PHA-Trypsin-Gemische in steigenden Konzentrationen den Lymphozytenkulturen zugesetzt Um eine Proteolyse von PHA auszuschliessen, wurden die Trypsinkonzentrationen so niedrig gehalten, dass die tryptische Aktivität durch die Antiprotease des PHA vollständig abgebunden werden konnte und somit keine freie Esterase nachweisbar war Wie Abbildung 2 zeigt, fuhrten niedrige Trypsinkonzentrationen zu einer Reduktion des Dosismaximums von PHA, während höhere Konzentrationen eine Verschiebung des Maximums bewirkten

Wirkung von Proteaseinhibitoren auf unstimulierte Lymphozyten Pflanzliche und tierische Proteaseinhibitoren aus Sojabohnen, Limabohnen, Pankreas und Ovomucoid zeigten in unstimulierten, serumhaltigen Lymphozytenkulturen keinen proliferationsfordernden, sondern eher einen leicht zytotoxischen Effekt (Tab II) Dieser war an der Abnahme der optischen Dichte (relatives Mass fur den Zellgehalt der Kultur) erkennbar

Effekt von Proteaseinhibitoren auf spezifisch und unspezifisch stimulierte Lymphozyten Wurden Lymphozyten mit PHA stimuliert, so war in Gegenwart von Serum mit SBI und mit Trasylol eine Zunahme der Lymphozyten-

Kulturen zugesetzt Es zeigte sich eine deutliche Abnahme der Lymphozytenstimulierung mit steigender Trypsinkonzentration (Abb 4) Dabei war es gleichgültig, ob das Serum-Trypsin Gemisch den Kulturen vor oder nach der Gabe von Tuberkulin oder gleichzeitig mit Tuberkulin zugesetzt wurde. Die Trypsinkonzentrationen wurden so niedrig gehalten, dass die Antiproteaseaktivität des Serums ausreichte und kein freies Trypsin vorhanden war

Esteraseaktivität in Lymphozytenextrakten und in zellfreien Kulturüberständen Extrakte von gewaschenen Lymphozyten enthielten eine esterolytische Aktivität gegenüber den synthetischen Estern BAEE und BTEE, die besonders in PHA stimulierten Kulturen auftrat und teilweise durch Trasylol hemmbar war (Tab III) Wurden Lymphozyten in Gegenwart von ^{14}C-Aminosäuren kultiviert und Extrakte aus diesen Lymphozyten mit Serum inkubiert, so zeigten die immunelektrophoretisch aufgetrennten Gemische autoradiographisch eine Markierung von a_2-Makroglobulin und zu geringem Teil auch von a_1-Antitrypsin Die Markierung nahm mit spezifischer und unspezifischer Stimulierung zu (Abb 5) Die Kontrollen – Lymphozytenextrakte ohne Serum und Extrakte und Serum, denen erst nach Abbruch der Kultur ^{14}C-Aminosäuren zugesetzt wurden – zeigten keine Markierung der genannten Proteine

Die Esteraseaktivität konnte auch in zellfreien Überständen nachgewiesen werden Wurden von serumhaltigen Lymphozytenkulturen nach 18 h Kulturdauer zellfreie Überstände gewonnen und durch Chromatographie über Sephadex G-25 von niedermolekularen Bestandteilen befreit, so war in den Überständen von tuberkulinstimulierten Kulturen eine esterolytische Aktivität gegen BAPNA nachweisbar, die deutlich höher lag als in Überständen unstimulierter Lymphozyten oder in den Kontrollen ohne Zellzusatz (Abb 6)

Diskussion

Das unspezifisch wirkende Mitogen PHA enthält Antiproteaseaktivität und inaktiviert Trypsin und andere proteolytische Enzyme [10, 11] Wir konnten zeigen, dass diese Antiproteaseaktivität in bestimmten PHA-Fraktionen vorhanden ist Nach Auftrennung von PHA über SE-Sephadex C-50 im diskontinuierlichen PHA-Gradienten nach WEBER [14] wurde das Antitrypsin in der zweiten Fraktion (pH 6 0) gefunden Auch in der nach GOLDBERG [3] durch Deproteinisierung mit Perchlorsäure gewonnenen Fraktion konnte Antiproteaseaktivität nachgewiesen werden Beide Frak-

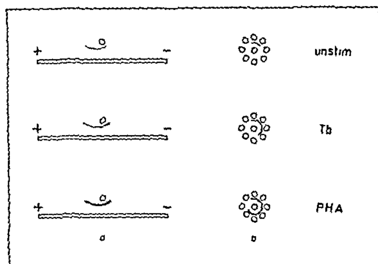


Abb. 5. Schematische Darstellung der Markierung von a_2-Makroglobulin durch Homogenate von mit [14]C-Aminosäuren inkubierten Lymphozyten und von Extrakten stimulierter und unstimulierter Lymphozyten mit Anti-a_2-Makroglobulinserum. Autoradiographie der Immunelektrophoresen (a) und der radialen Immundiffusion (b, Zentrum: Antiserum, aussen: abgestufte Verdünnungen der Lymphozytenhomogenate mit konstanter Menge Trägerserum)

tionen besassen eine deutliche lymphozytenstimulierende Wirkung. Da die Bindung von Trypsin an PHA zu einer ...
Das ...
... kung zukommt. Die Antiprotease- ... ist jedoch sicher nicht allein für den mitogenen Effekt verantwortlich, da auch nach Abbindung der Antiprotease mit Trypsin noch eine deutliche Lymphozytenstimulierung nachweisbar war. Die Wirkung von PHA auf Lymphozyten ist somit als ausserord...
Neben dem ...
seine ...
[5] ...
... ssen eine proliferationsfördernde ... nachgewiesen werden. Wie bereits in früheren Untersuchungen gezeigt wurde, besitzen a_2-Makroglobulin und a_1-Antitrypsin eine starke wachstumsfördernde Wirkung in Lymphozytenkulturen [6]. Ein ähnlicher wachstumsfördernder Effekt war auch mit SBI und zum Teil mit Trasylol nachweisbar. Die Antitrypsine des Serums und die genannten pflanzlichen und tierischen Antitrypsine wirken jedoch nur in stimulierten Kulturen,

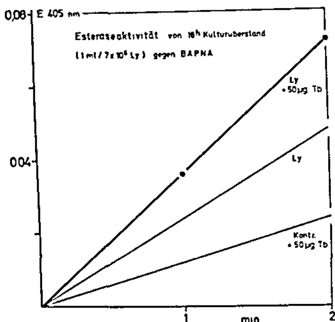


Abb. 6 Esteraseaktivität der Überstände von unstimulierten und mit Tuberkulin stimulierten Lymphozytenkulturen gegen BAPNA. Kontrolle: serumhaltiges Kulturmedium und Tuberkulin

d.h. sie sind im Gegensatz zu spezifischen und unspezifischen Mitogenen nicht in der Lage, das Lymphozytenwachstum auszulösen. Andererseits war es nicht möglich, die wachstumsfördernde Wirkung von Serum durch pflanzliche oder tierische Antiproteasen vollständig zu ersetzen. In allen verwandten Systemen zeigte SBI die stärkste Wirkung, während Trasylol nur in Gegenwart von Serum und mit PHA einen wachstumsfördernden Effekt aufwies. Ob das unterschiedliche Molekulargewicht, die unterschiedliche Spezifität oder andere Faktoren hierfür verantwortlich sind, ist nicht bekannt.

Unsere Ergebnisse ergänzen und unterstützen die vor kurzem beschriebenen Beobachtungen der wachstumsfördernden Wirkung von Antitrypsinen in Langzeitkulturen. So wurde von WALLIS *et al.* [13] die fördernde Wirkung von Serum in Affennierenzellkulturen auf die Antiproteaseaktivität zurückgeführt. LANDUREAU und STEINBUCH [7] konnten in Kulturen von Insektenfibroblasten einen Effekt von α_2-Makroglobulin und

Inter-α Antitrypsin nachweisen und vertraten die Ansicht, dass die Wirkung dieser Proteine auf der Inaktivierung lysosomaler Zellesterasen beruht Wir konnten in gewaschenen Lymphozytenextrakten und in zellfreien Kulturüberständen Esteraseaktivität nachweisen, die in stimulierten Kulturen deutlich höher lag als in unstimulierten Die Markierung von α_2-Makroglobulin und zu geringem Teil auch von α_1-Antitrypsin nach Zusatz von ^{14}C-markierten Lymphozytenextrakten macht eine Bindung der Esterasen an die genannten Serumproteine wahrscheinlich Die vorwiegende Bindung dieser Esterasen an α_2 Makroglobulin erklärt die Tatsache, dass trotz der hohen Antiproteaseaktivität der serumhaltigen Kulturüberstände Esteraseaktivität nachweisbar war Es ist bekannt, dass α_2-Makroglobulin nur die proteolytische Aktivität von Esterasen inaktiviert, während es die esterolytische Aktivität unbeeinflusst lässt [2]

Es kann aufgrund dieser Ergebnisse angenommen werden, dass während des Zellwachstums gebildete Lymphozytenesterasen an das umgebende Milieu abgegeben werden und dort von den Antitrypsinen des Serums gebunden werden Die Bindung dieser Lymphozytenesterasen an die Antiproteasen des Serums und an andere Antiproteasen pflanzlichen und tierischen Ursprungs ist für ein optimales Wachstum stimulierter Lymphozyten von entscheidender Bedeutung Ob dieser Effekt durch eine Inaktivierung zytotoxisch wirkender Esterasen oder durch eine Stabilisierung wachstums aktiver Esterasen zustande kommt, kann zum gegenwärtigen Zeitpunkt nicht entschieden werden

Zusammenfassung

Es wurde der Einfluss von Esterasen und Antiproteasen auf Lymphozytenkulturen untersucht Phythämagglutinin (PHA) und bestimmte PHA Fraktionen zeigten Anti trypsinaktivität Einige pflanzliche und tierische Antiproteasen wirkten wachstums fördernd auf stimulierte Lymphozytenkulturen ein Effekt der durch Vorbehandlung mit Trypsin deutlich vermindert wurde Zellhomogenate und zellfreie Überstände von stimu lierten Lymphozytenkulturen enthielten Esteraseaktivität Es konnte eine Bindung dieser Esterasen an Antiproteasen wahrscheinlich gemacht werden Vermutlich ist diese Bindung für die Aufrechterhaltung eines optimalen Lymphozytenwachstums von Bedeutung

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Acta haemat 46 294-306 (1971)

Association of Multiple Haematological Disorders (Acute Myeloblastic Leukaemia, Paraproteinaemia, and Thalassaemia) in a 46,XX/46,XXqi Female

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Abstract In a thalassaemic female carrying a 46,XX/46,XXqi mosaic, the occurrence of acute myeloblastic leukaemia was observed Karyotype analysis of several bone marrow aspirates disclosed, besides both normal metaphases and metaphases containing the iso chromosome a line characterised by a supernumerary chromosome in the C group (C trisomy) A clonal proliferation of the leukaemic line from normal precursors was suggested since no cells carrying the isochromosome and the C trisomy together were found In the course of the disease a remarkable plasma cellular proliferation brought about a hyperglobulinaemia of monoclonal aspect (IgG, λ type) Since the chromosomal analysis demonstrated that the presence of a C trison might also be present in the plasma cells, a close proliferative connection has bee put forward for the stem cells differentiating along both the granulocytic and th plasmacytic line

Key Words
Karyotype
Leukaemia
Multiple haemopathies
Paraproteinaemia
Plasma cells
Thalassaemia

Association of two or more haematological disorders has been frequently reported in the literature with implication of several pathogenetic perspectives and hypotheses In this connection the terms of 'mixed haematological tumours' or of 'polyblastic reticuloses' were introduced in order to focus the neoplastic involvement of different blood cell lines or the emergence of various morphological abnormalities concerning simultaneously or in turn several haemopoietic proliferative compartments [29] In this regard much emphasis has been given to the association of myeloid leukaemia with lymphoproliferative disorders [12, 15] or with Hodgkin's disease [11], or with monoclonal gammopathy [14, 23, 26] Although monoclonal gammopathy appears to be usually associated with pri-

mary plasma-cellular dyscrasias (myeloma and Waldenstrom's disease) this feature has been in the last few years demonstrated also in cases of acute lymphoblastic leukaemia terminating with myelomatosis [25 28] More recently ANDERSEN and VIDEBAECK [1] and KYLE *et al* [18] reported the development of acute myelomonocytic leukaemia in patients with multiple myeloma of long duration

Less frequently the association of systemic blood disorders with some autosomal or sex related chromosome abnormalities has been emphasised Apart from the high incidence of leukaemia in Down's patients relatively few other reports were dealing with the occurrence of genetically abnormal karyotypes and blood tumours in the same patient [17] We recently observed a patient showing multiple haematological disorders acute myeloblastic leukaemia paraproteinaemia (monoclonal gammopathy) and thalassaemia In addition the patient was carrying an abnormal karyotype showing a 46 XX/46,XXqi mosaicism The rarity of



Fig 1 Polymorphonuclear leucocytes illustrating different types of nuclear appendages *a b* large drumsticks *c d* normal-sized drumsticks May-Grünwald Giemsa × 1300

the association of these disorders and the possibility that a basic correla tion does exist among these situations prompt us to report this case

Case Report

The 56-year old female was first seen at our laboratory in June 1968 because of fatigue and fever of 6 months duration The family history was unremarkable except that she was born in the Po delta in an area associated with a high incid ence of thalassaemia in the population The patient had a delayed menarche (18 years) but no subsequent consistent abnormalities of the menses At the age of 21 she married and had two children both in good health The patient had thyphoid fever at the age of 22 and at the age of 48 she developed renal calculi

On admission the patient was complaining of fatigue, weakness and fever Physical examination revealed no hepatosplenomegaly or lymphoadenopathy The haematological data are given in table I In the peripheral blood 2% of blast forms were found and many neutrophils (26%) with a drum stick appendage larger than in the normal female were observed (fig 1) Buccal smears examination con firmed in many cells the presence of enlarged Barr bodies The blood film picture

Table I Haematological data obtained at various dates

	June 20 1968	July 18 1968	November 6 1968	June 28 1969	September 26 1969
Hb g%	7 2	7 1	7 8	6 4	5 2
RBC	3 000 000	3 060 000	3,200 000	2 940 000	2,360 000
WBC	1,500	1 400	1 800	1 400	13,200
Platelets	300 000	270 000	290 000	120 000	84 000
Differential %					
neutrophils	48	41	36	51	63
eosinophils	–	–	1	1	–
basophils	1	–	1	–	1
lymphocytes	48	49	43	37	18
monocytes	1	–	3	3	4
blasts	2	10	16	8	14
Reticulocytes ‰	22	18	16	18	12
Serum proteins g%	7 2	7 0	6 9	6 9	7 2
Electrophoresis %					
albumin	44	40	43	43	38
α_1 globulins	5	4	5	4	4
α_2 globulins	8	15	12	11	17
β globulins	12	13	13	9	8
γ globulins	31	28	27	33	33

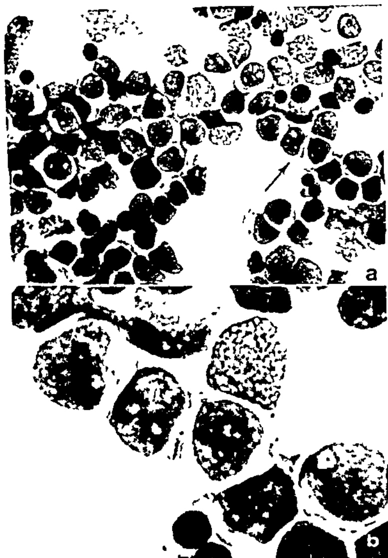


Fig. 2. *a* Bone marrow picture (June 1965) showing several myeloblasts. *b* Higher magnification showing a cell containing an Auer body in the cytoplasm (b). May-Grünwald-Giemsa. *a* × 460. *b* × 1,300.

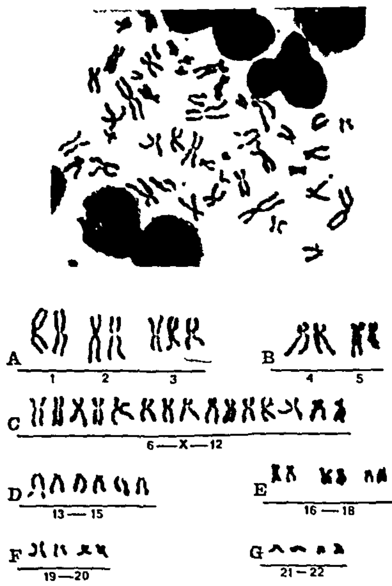


Fig. 3. Metaphase and karyotype containing 46 chromosomes (direct preparation from bone marrow aspirate). An abnormal metacentric chromosome similar in appearance to chromosomes of pair 3 is shown.

showed in addition several target cells, drop forms and schistocytes. Osmotic resistance was increased and electrophoresis of the haemoglobin disclosed an increased A_2 fraction.

Bone marrow examination showed a hypercellular specimen with predominance of the cells of the granuloblastic series. 32% of all nucleated cells were myeloblasts

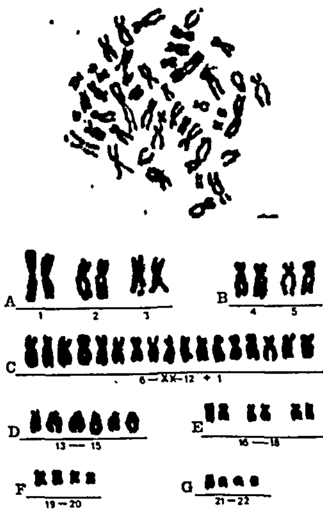


Fig 4 Metaphase and karyotype containing 47 chromosomes (direct preparation from bone marrow aspirate). An extrachromosome in C group is shown

Some of them showed occasional Auer bodies in the cytoplasm (fig 2). Plasma cells and lymphoid elements were inconspicuous.

Serum total protein content was 7.2 g/100 ml. No monoclonal bands were found on immunoelectrophoresis. The serum bilirubin was 0.30 mg/100 ml direct

Table II Chromosome distribution

Date	Preparation	< 40	41	42	43	44	45	46	47	47	Total	Remarks	Treatment
June 20 1968	bone marrow direct	1	–	–	–	–	2	4	16	–	23	46 XX/47 XX C+	–
July 2 1968	bone marrow, direct	4	–	–	–	–	2	9	10	–	25	46 XX/46 XXqi/ 47,XX C+	
July 2 1968	blood culture	1	1	–	–	–	–	28	3	–	33	46 XX/46 XXqi	–
June 9, 1969	bone marrow, direct	2	–	–	–	2	–	15	21	–	40	46 XX/46 XXqi/ 47 XX C+	prednisolone 6-MP
September 6 1969	bone marrow, direct	3	1	–	–	1	3	12	22	–	42	46 XX/46 XXqi/ 47,XX C+	prednisolone

and 0.90 mg/100 ml indirect Coombs test was negative. X ray film of the chest and the bones was normal

Chromosome studies from peripheral blood culture, according to MOORHEAD *et al.* [21], revealed in 71% of the euploid metaphases a normal karyotype. In the remaining metaphases an isochromosome for the long arms of the X was demonstrated. In direct preparations from bone marrow aspirates, the chromosomal analysis demonstrated besides normal metaphases, few metaphases with an isochromosome for the long arms of the X (fig 3) and several mitoses with 47 chromosomes (fig 4). These latter metaphases contained an extrachromosome in the C group. None of the examined cells showed the co-existence in the same plate of the isochromosome and the extrachromosome. No cells with XO complement were found. The results are summarised in table II

At this stage treatment was started of prednisolone (40 mg day) and transfusions were given. After her discharge, her conditions remained good until June 1969 when fever weakness and pallor recurred. On this second admission the patient was markedly anaemic. Hepatosplenomegaly could be demonstrated on physical examination. In this occasion the serum electrophoresis disclosed a quite narrow peak of γ globulins. This finding was confirmed by the results of immunoelectrophoresis and by the quantitative determination of the immunoglobulins by radial diffusion technique (IgG 2,700 mg%, IgA 200 mg%, IgM 110 mg%). Bence

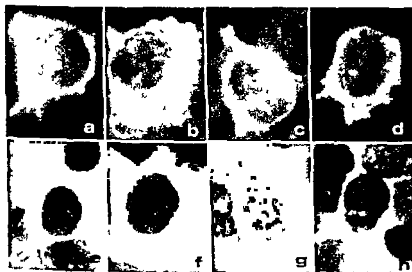


Fig 5. Immunofluorescence of bone marrow cells. *a, b, c, d* Plasma cells and Plasmablasts positive for IgG. *e, f, g, h* The same cells after staining of the preparation with May-Grünwald-Giemsa. × 1,235

Jones proteinuria was not found The serum paraprotein was classified as IgG, type λ

Bone marrow examination showed a still hypercellular specimen with myeloid/erythroid ratio 4 1 Granuloblastic series was mainly represented by immature elements, particularly myeloblasts An increased number of plasma cells and plasmablasts was also observed A study of bone marrow aspirates by the immunofluorescence technique disclosed an increased number of IgG positive plasma cells Many positive cells characterised by a round shaped aspect were classified as plasmablasts [20] Staining of the bone marrow smears after fluorescence studies with the Pappenheim method demonstrated clearly that the cells positive for IgG fluorescent antisera did not only belong to the plasmacytic type, but also to cells with 'lympho reticular' features (fig 5) Staining with fluorescent anti λ antiserum showed that several plasma cells and plasmablasts were markedly reacting

At this stage the patient was treated with prednisolone (40 mg/day) and with 6-mercaptopurine Late in September 1969 the patient suffered a generalised relapse of the leukaemic process with fever, hepatomegaly and splenomegaly Bone marrow biopsy showed a prominent plasma cellular and 'lympho-reticular' picture with decrease of both the erythroid and granuloblastic series A narrow band protein fraction was still visible on electrophoresis Proteinuria was absent The patient died at home on January, 18, 1970 of intercurrent broncho-pneumonia No *post mortem* examination was performed

Discussion

Although the association of multiple haematological disorders in the same patient has been reported frequently in the last few years, the peculiar features of this case deserve some further consideration Our patient showed the association of three basic disorders an abnormality of the karyotype with involvement of the sex chromosomes (X gonadal dysgenesis), a myeloproliferative disorder associated with the development of a paraproteinaemia, and the thalassaemia trait All these clinical features will be discussed separately

As far as the presence of a gonadal syndrome is concerned, it was unsuspected before examining the karyotype because of the absence of morphological malformations and the existence of a harmonious development of stature associated with normal secondary female sexual characters Apart from a delayed menarche, the patient had, in addition, two regular pregnancies As a matter of fact, gonadal syndrome may be extremely varied, because the degree of the dysgenesis may account for the intensity of the impairment of the function of the ovary According to German [13] although in the X gonadal dysgenesis most structurally ab-

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Abb 2a–c Blastenschub bei Osteomyelosklerose Peripherer Blutausstrich Pappenheim *b* Neben den Blasten sieht man Granulozyten mit gemischter Granulation sowie *a* Pseudo-Pelger Zellen mit eosinophiler oder *b c* neutrophiler Granulation

über 70% Paraleukoblasten Dieser Blastenschub war therapeutisch nicht beherrschbar Der Patient verstarb 2 Wochen nach der erneuten stationären Aufnahme Bei der Obduktion lag makroskopisch der Skelettbefund einer Osteomyelosklerose vor Die sehr stark vergrosserte Milz (Gewicht 3300 g) war von mehreren Infarkten durchsetzt Histologisch war eine diffuse leukotische Infiltration aus überwiegend unreifen Zellen im Markraum des Knochenmarks, in Milz und Leber nachweisbar

Zytologische und zytochemische Befunde

An peripheren Blutausstrichen wurden ausser der Pappenheim Färbung folgende Reaktionen ausgeführt

Toluidinblaufärbung bei pH 4,96, PAS-Färbung Sudanschwarz B Färbung α Naphthylazetat Esterase nach LÖFFLER Naphthol AS-Azetat Esterase nach LÖFFLER mit und ohne Hemmung durch Natriumfluorid Naphthol AS-D Chlorazetat Esterase nach BURSTONE in der Modifikation nach LEDER, Peroxydase nach SATO und SEKIYA, alkalische Phosphatase nach MERKER und HEILMEYER, saure Phosphatase mit dem Substrat Naphtol AS-BI Phosphat in der Modifikation nach LEDER

Bei Pappenheim Färbung sind in grosser Zahl atypische und polymorphe unreife Zellen mit pseudopodienartigen Plasmaprotrusionen und multiplen Nukleolen nachweisbar (Abb 2a, c) Unter den reiferen Granulozyten finden sich etliche Zellen mit einem unsegmentierten runden oder höchstens etwas eingebuchteten Kern (sog Pseudo-Pelger-Zellen vom homozygoten Typ) und mit eosinophil (Abb 2a) oder neutrophil granuliertem Plasma (Abb 2b, c) Ausserdem sind von der Reifungs-

über Oberbauchbeschwerden zu klagen hatte, bot bei der ersten stationären Behandlung in unserer Klinik im April 1968 folgende Befunde: Reduzierter Kräftezustand, befriedigender Ernährungszustand. Haut etwas blass, sichtbare Schleimhäute gut durchblutet. Keine Zeichen hämorrhagischer Diathese. Leber 2 Querfinger unter dem rechten Rippenbogen, Milz von derber Konsistenz handbreit unter dem linken Rippenbogen tastbar. Keine Lymphknotenschwellungen.

BSG 10/21 mm. Hämoglobin 13,66 g%, Erythrozyten 4,48 Mill./mm³, Hb_E 30,6 pg. Leukozyten 54 000/mm³, davon 3% Myeloblasten, 9% Promyelozyten, 23% Myelozyten, 16% Metamyelozyten, 25% Stabkernige, 20% Segmentkernige, 2% Eosinophile, 2% Lymphozyten, 2 orthochromatische Erythroblasten auf 100 weisse Blutzellen. Retikulozyten 7‰. Thrombozyten 160 000/mm³. Aktivität der alkalischen Leukozytenphosphatase erhöht. Beim zweimaligen Versuch der Sternalpunktion fiel eine äusserst harte Kortikalis auf. Bei kräftiger Aspiration war lediglich etwas Blut zu gewinnen, das in seiner Zellzusammensetzung weitgehend dem Ausstrich des peripheren Blutes entsprach. Histologischer Befund des Beckenkammtrepanates (Abb. 1): Breite und plumpe Spongiosabälkchen mit zungenförmiger Apposition von Knochensubstanz an den Wachstumsspitzen der Spongiosa und stellenweise mit verbreiterten Osteoidsäumen. Eingeengter Markraum mit vermehrtem Fasergehalt und erheblicher Verminderung des hämopoetischen Gewebes. Es handelte sich demnach um eine Osteomyelosklerose.

10 Monate später traten unter erheblicher Verschlechterung des Allgemeinbefindens eine Anämie (Hb 7 g%) und eine Thrombozytopenie (21 000/mm³) auf. Bei einer Gesamtleukozytenzahl von 138 000/mm³ fanden sich im Differentialausstrich

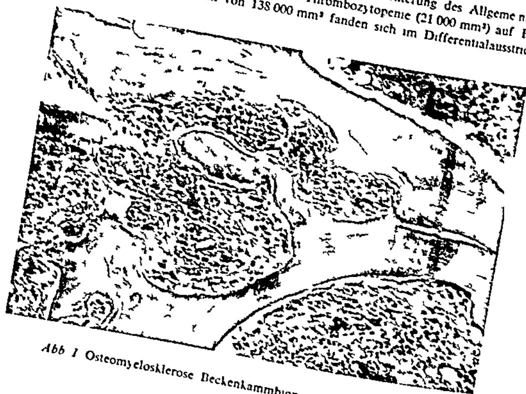


Abb. 1. Osteomyelosklerose. Beckenkammbiopsie. Hämatoxylin-Eosin

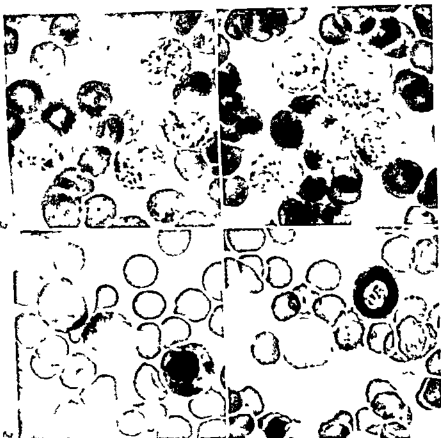


Abb. 5. Blastenschub bei Osteomyelosklerose. Peripherer Blutausstrich. Naphthol-AS-Azetat-Esterase. In Abbildung *b* links unterhalb der Bildmitte stark positiv reagierende Zytoplasmakugel

Abb. 6. Blastenschub bei Osteomyelosklerose. Peripherer Blutausstrich. *a* Naphthol-AS-D-Chlorazetat-Esterase. *b* Alkalische Phosphatase. Beide Fermente sind mit hoher Aktivität im Plasma von Pseudo-Pelger-Zellen enthalten. Blasten reagieren negativ

sten lassen zarte Plasmagranulationen oder auch dicke Granula und kleine Plasmaschollen erkennen (Abb. 3b).

α-Naphthylazetat-Esterase ist in allen Blasten nachweisbar, und zwar überwiegend mit schwacher Reaktion des ganzen Plasmasaums. Zusätzlich sieht man in vielen Zellen kleine umschriebene paranukleare Ver-



Abb 3 Blastenschub bei Osteomyelosklerose Peripherer Blutausstrich *a* Toluidinblau *b* PAS

Abb 4 Blastenschub bei Osteomyelosklerose Peripherer Blutausstrich *a* Naphthylazetat Esterase

stufe des Myelozyten ab einige Zellen mit gemischter neutrophiler, eosinophiler und basophiler Granulation zu sehen (Abb 2b)

Mit Toluidinblau stellen sich Zytoplasmaausstulpungen und Nukleolenpolymorphie der Blasten besonders deutlich dar (Abb 3a) Bei der PAS Farbung reagieren die neutrophilen Granulozyten positiv, zum Teil allerdings viel schwacher als normalerweise und nur in Form einer zarten peripheren Granulation Am kraftigsten farbt sich das Plasma der Pseudo Pelger Zellen Ein Teil der Blasten bleibt ungefarbt Andere Bla-

Mitunter ist das Reaktionsprodukt auch zu einem paranukleären, vorzugsweise in einer Kernbucht gelegenen Bezirk verdichtet.

Diskussion

Über den terminalen Blastenschub einer Osteomyelosklerose ist als seltenes Ereignis in der Literatur wiederholt berichtet worden [2, 4, 6, 8, 9, 17, 22, 24, 30—35, 37]. Bis zu dieser Phase, die stets klinisch mit einer erheblichen Verschlechterung einhergeht und meist unwiderruflich das therapierefraktäre Terminalstadium einleitet, unterscheidet sich der Krankheitsverlauf von dem anderer Osteomyelosklerosen gewöhnlich nicht. Gelegentlich wird auf einen verkürzten, klinisch maligneren Verlauf hingewiesen [30, 35].

Falls die Osteomyelosklerose als Grundleiden nicht bereits durch eindeutige Vorbefunde bekannt ist, bereitet die richtige Diagnose zu diesem Zeitpunkt Schwierigkeiten. Insbesondere ist die Differenzierung vom Blastenschub einer chronischen Myelose schwer bzw. unmöglich. Soweit Sternalmark überhaupt durch Punktion zu gewinnen ist, kann es völlig von der unreifzelligen Wucherung beherrscht sein, ähnlich wie alle extramedullären Organ- oder Hautinfiltrate. Die alkalische Granulozytenphosphatase kann in diesem Stadium zur Differentialdiagnose nicht mehr beitragen, da auch während des Paramyeloblastenschubs einer chronischen Myelose die zuvor erniedrigte Aktivität rasch ansteigt, mitunter sogar auf stark erhöhte Werte [7, 15, 21, 29]. Selbst der Nachweis des Philadelphia-Chromosoms ist nicht in jedem Falle zuverlässig [17, 24], da es sowohl Ph¹-negative chronische Myelosen [31] als auch seltene Ph¹-positive Osteomyelosklerosen gibt [22, 23]. Entscheidende diagnostische Bedeutung kommt dem histologischen Befund des Beckenkammtrepanats zu.

Der Blastenschub dokumentiert die wesensmässige Verwandtschaft der Osteomyelosklerose nicht nur mit der chronischen Myelose, sondern auch mit anderen Varianten des myeloproliferativen Syndroms wie der Polycythaemia vera, bei der ebenfalls terminale Blastenschübe vorkommen [2, 30, 31, 36]. Aus klinischer Sicht kann dieses Phänomen daher ebenso als Bestätigung der Konzeption DAMESHEKS vom myeloproliferativen Syndrom gelten wie die zahlreichen gesicherten Übergänge von chronischer Myelose, Polycythaemia vera oder essentieller Thrombozythämie in eine Osteomyelosklerose [2, 3, 4, 17, 22, 30–33] oder die



Abb 7 Blastenschub bei Osteomyelosklerose Peripherer Blutausstrich Saure Phosphatase

dichtungszonen des farbigen Reaktionsprodukts (Abb 4a) Bei einem wesentlich kleineren Teil der Blasten findet sich eine hohe Enzymaktivitat in kappenformig angeordneten paranukleären Bezirken (Abb 4b) Naphthol-AS-Azetat-Esterase ist in allen Blasten enthalten Das Reaktionsprodukt ist granular im Plasma verteilt und uberlagert teilweise den Kern (Abb 5) Mitunter sieht man von den Zellen losgeloste Zytoplasmakugeln mit hoher Fermentaktivitat (Abb 5b) Durch Zusatz von Natriumfluorid zum Inkubationsgemisch wird das Enzym vollig gehemmt

Naphthol-AS-D Chlorazetat-Esterase ist in den Blasten nicht darstellbar Neutrophile Granulozyten reagieren in ublicher Weise positiv, am starksten die Pseudo-Pelger-Zellen (Abb 6a) Prinzipiell gleichartige Ergebnisse sind beim Nachweis der Peroxydase und bei Anfarbung mit Sudanschwarz B zu erzielen Ebenfalls negativ reagieren die Blasten beim Nachweis der alkalischen Phosphatase (Abb 6b) In der Restgranulopoese besteht insgesamt eine massig erhohte Aktivitat dieses Ferments, wobei auch die Reaktionsklassen 4 und 5 nach MERKER und HEILMEYER vorhanden sind Diese hohen Aktivitatsstufen lassen sich vor allem in Pseudo-Pelger-Zellen nachweisen (Abb 6b)

Saure Phosphatase ist mit hoher Aktivitat als fein- bis grobgranularer Niederschlag der haufig auf einer Zellseite zusammengedrangt angeordnet ist und dann dem Kern kappenartig aufsitzt, lokalisierbar (Abb 7)

ger Zellen vom homozygoten Typ, wie sie gelegentlich bei Hämoblastosen vorkommen [18]. Zytochemisch ist auffällig, dass die für reife Granulozyten charakteristischen Reaktionen in diesen Zellen stärker positiv ausfallen als in den normal segmentierten Leukozyten.

Bemerkenswert ist ferner eine seltene Granulationsanomalie, die bei unserem Patienten an einigen Leukozyten gleichzeitig mit dem Einsetzen des Blastenschubs erstmals auftrat. Es handelt sich um Zellen mit einer gemischten neutrophilen, eosinophilen und basophilen Granulation. Dieselbe Beobachtung machten BOURONCLE und DOAN [4] in der Blastenphase einer Osteomyelosklerose.

Zusammenfassung

Es wird über einen 25jährigen Patienten berichtet, der nach vierjährigem Krankheitsverlauf einer Osteomyelosklerose an den Folgen eines akuten Blastenschubs verstarb. Zytochemische Untersuchungen der pathologischen Blutzellen ergaben Befunde, die von denen aller näher definierbaren Formen unreifzelliger Hämoblastosen abwichen, so dass eine Zuordnung der Blasten zu einer bestimmten Zellreihe nicht gelang. Die Möglichkeit wird diskutiert, dass eine genetisch inhomogene Blastenpopulation aus entdifferenzierten Zellformen der weissen und roten Reihe vorliegen könnte.

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häufigen Zwischenformen, die sich klinisch und morphologisch auch bei Anwendung diffiziler Untersuchungsmethoden nicht klassifizieren lassen [17, 30, 31] In der noch häufig diskutierten Frage nach der biologischen Wertigkeit der Osteomyelosklerose spricht das Vorkommen der Blastenphase gegen die Annahme der narbigen Defektheilung einer serosen Myelitis [27] und für die Deutung als leukämieäquivalente Neubildung des Markorgans, die aufgrund der morphologisch dominierenden bindegewebigen Stromaentwicklung als «Scirrhus des myeloischen Parenchyms» interpretiert werden kann [17]

Bezüglich der näheren Typisierung der Blasten können zytochemische Methoden weiterhelfen die sich bei der Klassifizierung der einzelnen Formen unreifzelliger Leukosen bewährt haben [1, 10, 12, 13, 16, 19 (dort weitere Lit) 20, 25]

Legt man die bei derartigen Untersuchungen in den letzten Jahren empirisch gefundenen Massstabe an, so zeigt sich in unserem Falle, dass sich die Blasten aufgrund ihrer baustein- und fermentzytochemischen Merkmale nicht in das übliche Konzept der unreifzelligen Leukosen einordnen lassen Während manche Einzelbefunde auf eine myeloische oder monozytare Differenzierungsrichtung hinzudeuten scheinen, finden sich an einem Teil der pathologischen Zellen auch gewisse Parallelen zum Bilde des Paraerythroblasten [1, 5, 7, 11], insbesondere hinsichtlich der Aktivität und Lokalisation der sauren Phosphatase Zytochemisch zeigt sich an den Blasten im ganzen eine Befundvielfalt, die im panoptisch gefärbten Präparat überhaupt nicht zur Darstellung kommt Die grossen morphologischen Variationsmöglichkeiten myeloproliferativer Erkrankungen können sich demnach auch im terminalen Blastenschub aussern

Diese Befunde legen den Gedanken nahe, dass die Blasten aus verschiedenen Zellpopulationen hervorgegangen sein konnten, zumal bekannt ist, dass in der Terminalphase einer Osteomyelosklerose nicht nur das Bild einer unreifzelligen Leukose, sondern auch einer Erythroblastose bestehen kann [30, 32, 34] In diesem Zusammenhang sei an die Auffassung von ROHR [26] erinnert, wonach der Paraleukoblast ein Sammelbegriff für genetisch verschiedene Endformen ist, die durch Entdifferenzierungsvorgange aus unterschiedlichen Zellinien entstanden sind Nur so ist es auch verständlich, dass Paramyeloblastenschübe bei Erythrämie [5, 17] oder ein zytochemisch gesicherter Paraerythroblastenschub bei chronischer Myelose [11] beobachtet wurden

Abschliessend soll auf einige Besonderheiten der Restgranulopoese hingewiesen werden In relativ hoher Zahl finden sich sog Pseudo-Pel

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Demonstration of enzymic activities The water used was distilled and deionized; e acet ne was redistilled Before being incubated the tissue preparations were oped in cold acetone for 5 minutes to remove lipids and then in 2 changes of illed 0.1 M phosphate saline pH 7.5 for a total of 5 minutes to remove water luble end genous substrates

a) Some sections were then incubated to demonstrate the location and degree of tivity of Δ⁵ 3β hydroxysteroid dehydrogenase an enzyme occurring early in the thway for steroid hormone biosynthesis (Samuels 1960 Rubin et al 1963 Deane & 'on 1965 The medium is given in Table 2 The control medium was similar except at it lacked DHA Generally 4 to * coverslips were placed in a Columbia staining ar and incubations were carried out for 1 h and 5 h at 40 C.

Table 2

Composition of medium for demonstrating the activity of Δ⁵ 3β hydroxysteroid dehydrogenase

	Amount
ton c saline buffered with 0.1 M phosphate pH 7.5	4.0 ml
Nitro blue tetrazolium (NBT Sigma 1 mg ml H₂O	2.0 ml
Dehydroepiandrosterone DHA Sigma 0.5 mg ml acetone	0.5 ml
Nicotinamide adenine dinucleotide (NAD or DPN Boehringer) 6 mg ml H_2O	1.0 ml

b) For demonstrating the activity of NADH-tetrazolium reductase otherwise known as NAD diaphorase a total of 7.0 ml of medium contained buffer saline and NBT as were plus 3 mg NADH (or DPNH Incubations usually ran for ½ h

c) For NADPH-tetrazolium reductase activity the medium contained 3 mg NADPH (TPNH and incubations generally lasted 1 h. This enzyme may be considered an indicative of the hexose monophosphate pathway which is presumably involved in steroid hormone biosynthesis (Hayano & Dorfman 1962 Deane et al 1962)

After the incubation the preparations were washed fixed in ethanol formalin washed again and mounted in Gurr's Water Mounting Medium

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ner zahlreichen Bezüge auf klinische Probleme auch dem Assistenten und dem praktischen Arzt wertvolle Anregungen. F GLOOR, *St Gallen*

A KLEINZELLER, G F SPRINGER and H G WITTMAN (ed) **Molecular Biology, Biochemistry and Biophysics, vol 9 The Genetic Markers of Human Immunoglobulins.** Springer, Berlin/Heidelberg/New York 1970 XII + 152 pp., 8 fig. Cloth DM 42.–, US $ 11 60

Immunoglobulin polymorphism expresses the functional heterogeneity of antibodies. Protein structure is genetically determined. The study of genetic markers of immunoglobulins provides insight into immunoglobulin differentiation and its control and testifies that a major part of the immunoglobulin molecule is under Mendelian control The data about the genetic markers are widely scattered in various journals It is fortunate that Prof GRUBB who discovered the Gm system undertook the considerable task to assemble the information about human immunoglobulin markers in a concise and clear form His review not only is very complete but puts the available information into the context of modern biological thinking taking into account chemical evolutionary, practical and other aspects. After an introduction, basic data concerning the genetic markers are given, including techniques of detection. The next chapters deal with the chemical correlates followed by the formal genetics of the Gm and Inv systems. The human anti-human γ-globulins are discussed, including their possible role in the body The concluding chapter is about the selection and the control of immunoglobulin structure. In a most useful appendix there is a compilation of data concerning the genetic factors, followed by the references and a complete subject index. This review by Prof GRUBB is very stimulating reading for every biologist, and a most useful reference source for anyone working in this field. T. L. VISCHER, *Basel*

J M THOMSON **A Practical Guide to Blood Coagulation and Haemostasis.** Churchill London 1970. 220 pp £ 2.80

Numerous books on blood coagulation and haemostasis were written during the past few years. Unfortunately the available modifications of methods for evaluation of disorders of blood coagulation and haemostasis are far from being internationally comparable. Each author tends to emphasise the methods to which he is accustomed through his own experience Some national habits are deeply entrenched in this field The book by JEAN THOMSON reflects the British way of studying patients, which in many respects should be internationally followed However, the term 'practical guide' is not entirely adequate. The recommended screening programs are rather complex and imply the use of comparatively large amounts of plasma. Theoretical aspects are adequately covered. Some helpful tables and figures are included.

The final impression of this 'practical guide' is a mixed one too many details for the small district hospital type of laboratory, too little and incomplete information for the experienced worker were compiled in this volume Recent discoveries on abnormal (structural) variants of coagulation factors, particularly fibrinogen, are not mentioned The concept of von Willebrand's disease (p 65) is misleading CRAIG *et al* [1963] found that a transfusion of blood from a patient with clas-

Bono Hak Hyun A Filmstrip Presentation. Hematology. A Morphologic Study.

Es handelt sich bei diesem neuartigen Werk um eine Sammlung von ca 300 farbigen Mikrophotographien auf 3 Filmbandern (35 mm) mit einem Kommentar in Form eines kleinen (11/13 cm), 84 Seiten umfassenden Heftes Dem Referenten stand lediglich ein kurzer Filmstreifen mit 6 ausgewahlten Bildern sowie der Kommentar zur Verfugung Nach den wenigen Beispielen zu schliessen, sind die Qualitat der Aufnahmen sowie die Auswahl der Ausschnitte vorzüglich Der erste Filmstreifen umfasst die Veranderungen des roten Systems Neben Blut- und Knochenmarksausstrichen werden besonders auch Knochenmarksschnitte berücksichtigt Der Kommentar beschrankt sich in der Regel auf eine knappe Beschreibung des Bildes, welche gewisse Basalkenntnisse (Begriffe usw) voraussetzt Gelegentlich sind stichwortartig auch klinische Daten erwahnt Die Dokumentation ist sehr reichhaltig, werden doch zum Beispiel 18 Aufnahmen zum Kapitel megaloblästäre Anamie gezeigt. Das zweite Filmband umfasst die malignen Hamoblastosen, das dritte schliesslich Bilder von Knochenmetastasen, megakaryozytaren Storungen, Granulomen, Speicherkrankheiten usw Auch Bilder von Agranulozytose, von reaktiven Lymphozytosen etc finden sich auf diesem Filmstreifen

Selbstverstandlich ist die Beurteilung eines Werkes schwierig, wenn die Hauptsache, nämlich das Bildmaterial, fehlt Es scheint sich jedoch um eine vielseitige, instruktive Auswahl zu handeln, die sich vorzuglich zum Selbststudium eignet Besonders nützlich dürfte sie fur Studenten oder Ärzte sein, welche bereits gewisse Kenntnisse in Morphologie und systematischer Hamatologie besitzen, diese aber wieder aufzufrischen oder zu erweitern wünschen Solche Hilfsmittel werden in Zukunft mehr und mehr benotigt werden, da sich nicht mehr jeder Student selbst eine Sammlung von Praparaten anlegen kann U Bucher, *Bern*

W Doerr **Spezielle Pathologische Anatomie I.** Heidelberger Taschenbücher, Vol 69 Springer, Berlin 1970 XVII +403 pp, DM 6 80/US$ 1 90

In der Reihe der Heidelberger Taschenbucher wird von Wilhelm Doerr in drei Banden die Allgemeine und Spezielle Pathologische Anatomie abgehandelt Der vorliegende Band «Spezielle Pathologische Anatomie I» behandelt das Herz und Gefasssystem, die Luftwege und Atmungsorgane, das Blut und die blutbildenden Organe sowie die Nieren und die ableitenden Harnwege Die Darstellung ist aus den Heidelberger Vorlesungen des Autors hervorgegangen und lasst die grosse Erfahrung des langjährigen Hochschullehrers erkennen Die Fülle des dargebotenen Stoffes ist erstaunlich Trotzdem wird erfreulicherweise eine allzu starke Schematisierung vermieden Besonders wertvoll sind die kritischen Bemerkungen zu den noch offenen Problemen, die den Leser zum eigenen Nachdenken anregen Es fehlt auch nicht an kurzen Hinweisen auf historische Daten In einzelnen Kapiteln wie demjenigen der Arteriosklerose wird das personliche Kolorit des Doerrschen Arbeitskreises sichtbar Das fast vollständige Fehlen von Abbildungen und Schemata dürfte dem Anfänger Schwierigkeiten bereiten Das Taschenbuch ist in erster Linie fur den Studenten in klinischen Semestern gedacht Es gibt jedoch wegen sei

Acta haemat 46 321-325 (1971)

Temporal Variation in Mitotic Index of Phytohaemagglutinin Stimulated Human Lymphocytes

D J PRICE and J TIMSON

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Abstract Data is presented which indicates that over a period of time in a given individual the response to PHA stimulation as measured by lymphocyte transformation is constant The mitotic rate in these cells, however, shows great variation suggesting that DNA synthesis is necessary but not sufficient for mitosis

Key Words
Lymphocyte culture
Lymphocyte mitosis
Phytohaemagglutinin

The phytohaemagglutinin (PHA) stimulated human lymphocyte has been widely used to screen compounds for their ability to affect the mitotic rate [e g 1 and 2] and to cause chromosome damage [3, 4] Some of the problems involved in obtaining reproducibility in this system have been reviewed [5] It has been reported that individuals respond differently to PHA stimulation [6] and have different mitotic timing [7] What does not seem to have been previously reported is the variation in response of an individual when several blood samples are taken over a period of several months from that individual

The experiments reported here were undertaken to investigate this variation in response in terms of morphological transformation and the rate of mitosis

Materials and Methods

The method used for culturing the lymphocytes is based on that of Moorhead *et al* [1] and was as follows 20 ml samples of venous blood were obtained from healthy volunteers and mixed immediately with anticoagulant (heparin in dextran) in a sterile container The erythrocytes were allowed to settle at 37°C and the supernatant plasma and leucocytes were drawn off 1.5 ml of the cell/plasma suspension was made up to 10 ml with TC199 culture medium (Glaxo) and 2 drops of reconstituted PHA P (Difco) were

sic hemophilia corrected the bleeding time and the factor VIII level in patients with von Willebrand's disease, but blood from von Willebrand's disease does not restore the factor VIII level in haemophilia *Therefore, it is patent that blood from a apparent severe factor VIII deficiency in von Willebrand's disease must never be used as substrate plasma in the laboratory diagnosis of haemophilia* (italics mine) Does the author imply that haemophilic plasma creates factor VIII *in vitro*?

The discerning reader may find some useful details in this small volume, such as on quality controls of thromboplastins and inhibitor assays However, the reviewer would not recommend its indiscriminate use as 'practical guide' for the small hospital laboratory E A BECK, *Bern*

Varia

Viviana Luckhaus Foundation

The Fundación Viviana Luckhaus has instituted the International Prize Fundación Viviana Luckhaus, 1972 The Prize is intended to honour a report of original research related to blood platelets (morphology, physiology, biochemistry pathology, etc) and/or their relationship to thrombosis and blood vessels, and to promote communication and interchange between research workers in different parts of the world

For further information and rules for the 1972 contest apply to Dr EDGARDO S SACK, Fundación Viviana Luckhaus, Hospital Juan A Fernández Cerviño 3356 *Buenos Aires* (Argentina)

are all low indicating a reasonably consistent response However, since transformation is essentially complete at 48 h, variation may be masked since if the transformation process was slowed down by up to 24 h, the % transformation at the end of 72 h would be the same

The mitotic index however, is much more variable Different individuals have more variable means as might be expected, but there is also a large variation in the rate of mitosis within an individual The range is wider and the standard deviations are large, having an average of approximately 12. Thus the mitotic index of a person varies from sample to sample over a period of time It has been suggested that the cell cycle time is constant for an individual [7] but the results outlined above suggest that this may not be so

Differences in the culture media used may be ruled out as a cause of the variation because it was found that when samples from different individuals were cultured simultaneously using the same batch of culture media high values in the mitotic index of one individual were not associated with high values of the other individual [5] Neither were low values of one person associated with low values of another High and low values in the mitotic index of a person were randomly distributed over the 15-month period and consecutive samples taken 1 week apart from one individual cultured with the same batch of culture media PHA, etc did not necessarily give the same mitotic response It is possible that subclinical infections could explain some of the variation but this seems unlikely to be a major factor considering both the magnitude and frequency of the variation

Addition of PHA to cultures of lymphocytes does not affect cells already undergoing DNA synthesis [9–11] It causes synthesis of DNA and the onset of mitosis in a proportion of cells [9 10] which have been identified as almost all being blast cells [12]. Hence, since transformation is relatively constant within an individual it is possible that DNA synthesis which is necessary for mitosis to occur is also relatively constant Events occurring after the synthesis of DNA in the G_2 phase of the cell cycle may however, be more variable and be a cause of the variation in mitotic rate Synthesis of enzymes needed for the formation and functioning of the mitotic spindle for example may be particularly important in this respect

If the cell cycle is itself constant then the variation may be due to variation of factors present in the plasma which the cultures contain Humoral substances which increase the rate of lymphocyte production are found in conditions of leucopenia [13] are present when lymphocytosis occurs [14 15] and occur in the thymus [16] It has been suggested that all these substances

added to each culture which was then incubated at 37°C for 72 h At the end of this period 0.2 ml of demecolcine (Colcemid®) at a concentration of 1 mg in 100 ml was added and the cultures incubated for a further 2 h The cells were then centrifuged (1,000 rpm for 10 min) the supernatant discarded and the cells resuspended in hypotonic saline for 15 min at 37°C After this period they were fixed in 2 changes of acetic alcohol (1 part glacial acetic acid 3 parts ethanol) and resuspended in 45% acetic acid to give an opalescent suspension The cells were dropped onto cold slides, air dried, and stained with 10% Giemsa buffered at pH 6 4

One thousand cells per culture were examined and the number of cells in mitosis noted This number is defined as the mitotic index The first 200 cells examined were also classified into transformed and untransformed cells to give the % transformation rate

Samples were taken at random intervals over a 15 months period from 3 males and 3 females When the samples were taken the donors were healthy and had no recent history of infection They were not taking any drugs and had not been exposed to radiation

Results

Table I shows the number of samples taken from each individual, the mean transformation and mitotic rate for each individual together with the standard deviations and ranges for these values

Discussion

As can be seen from table I, the average transformation rate of all 6 individuals tested falls between 62 and 66% The maximum variation in transformation rate of an individual was 12% but the standard deviations

Table I The variation within an individual of response to PHA stimulation when measured by the transformation and mitotic rates (72 hour cultures)

Volunteer	Sex	Samples	% transformation			Mitotic index		
			mean	SD	range	mean	SD	range
1	male	24	65 8	4 85	53–73	26 3	13 06	4–62
2	male	8	65 6	5 89	56–74	37 4	18 63	10–72
3	male	5	62 4	3 26	59–68	24 8	13 79	6–45
4	female	13	65 6	5 24	56–71	25 2	13 00	7–50
5	female	7	65 1	3 76	60–70	34 6	11 79	23–60
6	female	5	65 2	4 96	59–71	20 0	3 58	11–24

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are lymphopoietin and has been shown that lymphopoietin in chronic lymphatic leukaemia serum stimulates mitosis in normal PHA stimulated human lymphocytes [17] Furthermore, control of the production of lymphopoietin by negative feedback mechanisms and the possible existence of more than one type of lymphopoietin have been suggested [17] Hence it is possible that the variation within an individual of mitotic rate reported here is a result of variation in the amount of lymphopoietin and other regulators of mitosis present in the plasma

Since at present lymphocyte culture without plasma is not satisfactory such variation is inevitable This emphasises the importance of control cultures being grown for each blood sample It is then possible to compare results in terms of a function of the control value, e g , percentage of control

Finally, this variation may be of importance when testing compounds for their ability to affect the rate of mitosis since a person whose mitotic rate is high may respond differently to a compound than when his mitotic rate is low

Acknowledgement We wish to thank the Smith Kline and French Foundation for financial support

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zytentransformation durch einfache Metallverbindungen beschrieben [4, 17, 25, 27] und wir können in der vorliegenden Arbeit nachweisen, dass auch bei Verwendung eines derartigen Modells ein wesentlicher Unterschied zwischen gesunden Versuchspersonen und Patienten mit Lymphogranulomatose und chronischer *lymphatischer Leukämie besteht*.

Patienten

Untersucht wurden 28 nicht ausgesuchte poliklinische und stationäre Patienten mit Lymphogranulomatose im Alter von 20 bis 74 Jahren. Davon hatten 5 Patienten zu keinem Zeitpunkt eine spezifische zytostatische und/oder radiologische Therapie erhalten, bei allen übrigen Patienten lag die zuletzt durchgeführte Therapie mindestens 4 Wochen zurück. Die Diagnose war in jedem Fall durch zytologische und/oder histologische Untersuchung gesichert. Eine Stadieneinteilung bei den Patienten erfolgte nach dem heute allgemein anerkannten Schema [23]. Ausserdem wurden die Patienten, die sich zum Zeitpunkt der Untersuchung in einem aktiven Stadium der Erkrankung befanden, denen gegenübergestellt, die sich bei Fehlen klinischer, hämatologischer und radiologischer Symptome in Remission befanden (Tab. I).

Wir untersuchten weiterhin 27 Patienten mit chronischer lymphatischer Leukämie im Alter von 55 bis 81 Jahren, bei denen zum Zeitpunkt der Untersuchung keine Therapie durchgeführt wurde. Die letzte antileukotische Therapie lag mindestens 2 Monate zurück. 21 Patienten hatten periphere Leukozytenzahlen zwischen 10 000 und 100 000/mm³, bei 6 Patienten lagen die Werte über 100 000/mm³.

Als Kontrollkollektiv dienten 23 Versuchspersonen, bei denen keine Anhaltspunkte für irgendwelche Erkrankungen bestanden.

Tabelle I. Einteilung der Patienten mit Lymphogranulomatose im Hinblick auf das Erkrankungsstadium

Stadium	Zahl der Patienten	
	in Remission[1]	aktives Stadium
I	3	–
II	5	3
III	7	9
IV	–	1
Total	15	13

[1] Die Zuordnung in die Stadien I–IV bei den Patienten, die sich als Folge einer Therapie in Remission befanden, erfolgte nach den vor dieser Therapie bestehenden Kriterien.

Acta haemat 46 326–337 (1971)

Stimulation peripherer menschlicher Lymphozyten *in vitro* durch Zn^{2+} bei Patienten mit Lymphogranulomatose und chronischer lymphatischer Leukämie[1]

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Abstract The response of peripheral lymphocytes to Zn^{2+} *in vitro* (blast cell count and ^3H thymidine uptake) was found to be greatly diminished in patients with Hodgkin's disease and chronic lymphocytic leukemia, when compared with normal volunteers This seems to reflect a cellular defect of the lymphocytes In *Hodgkin's disease* the degree of stimulation was independent of the absolute lymphocyte count in the peripheral blood but was higher in patients in remission after therapy than in those with an active disease The response of the lymphocytes in *chronic lymphocytic leukemia* could be improved by culturing the cells at a higher density than is normally used

Key Words
Chronische lymphatische Leukämie
Lymphogranulomatose
Lymphozytenkultur
Zinc stimulation

Die zentrale Rolle des Lymphozyten bei vielen Immunreaktionen gilt heute als gesichert [7] Erkrankungen, die mit einer Beeinträchtigung des lymphatischen Systems einhergehen, wie z. B die Lymphogranulomatose und die chronische lymphatische Leukämie, weisen einen Defekt der zellulären Immunität auf, der sich unter anderem in einer verzögerten Abstossung homologer Hauttransplantate und in einer Beeinträchtigung der Kutanreaktion auf Antigene äussert [1, 2, 16]

Es ist weiterhin gezeigt worden, dass Patienten mit verschiedenen lymphoproliferativen Erkrankungen eine stark reduzierte Transformationsfähigkeit der peripheren Lymphozyten *in vitro* unter der Einwirkung von Phytohämagglutinin und spezifischen Antigenen aufweisen [3, 11, 12, 13, 19, 22, 24, 28] Kürzlich wurde eine unspezifische Lympho-

[1] Die Autoren bedanken sich bei Frau G Böchert für ausgezeichnete technische Assistenz.

zytentransformation durch einfache Metallverbindungen beschrieben [4, 17, 25, 27] und wir können in der vorliegenden Arbeit nachweisen, dass auch bei Verwendung eines derartigen Modells ein wesentlicher Unterschied zwischen gesunden Versuchspersonen und Patienten mit Lymphogranulomatose und chronischer lymphatischer Leukämie besteht.

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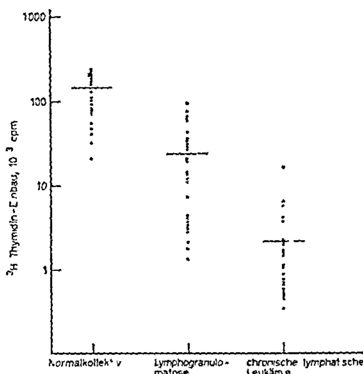


Abb 1 Zinkstimulation peripherer Lymphozyten bei gesunden Kontrollpersonen, Patienten mit Lymphogranulomatose und chronischer lymphatischer Leukämie (^{3}H-Thymidin Einbau nach 144 h) Jeder Punkt repräsentiert eine Versuchsperson, die Horizontallinien geben die Mittelwerte an

Bei allen untersuchten Patienten und Normalpersonen wurden vor der Untersuchung die wichtigsten hämatologischen Daten bestimmt

Lymphozytenkultur

Die Methode der Lymphozytenkultur ist an anderer Stelle ausführlich beschrieben worden [18] Wir verwendeten heparinisiertes Venenblut, das mit 6% Dextran 60 min sedimentiert wurde Als Kulturmedium diente Eagles Minimal Essential Medium Der normale Kulturansatz enthielt $1,0 \times 10^6$ Leukozyten/ml sowie 30% autologes Plasma (in einigen Experimenten blutgruppengleiches homologes Plasma) Die Zellen wurden in 16×125 mm grossen Plastikrohrchen bei 37 °C für 144 h inkubiert (5% CO_2 in Luft), in einigen Versuchen betrug die Inkubationszeit bis zu 240 h. Zinkchlorid wurde zu Versuchsbeginn zugesetzt, und zwar wurden in jedem Versuch 3 Zinkkonzentrationen getestet ($2,5$, $3,0$, $3,5 \times 10^{-4}$ M)

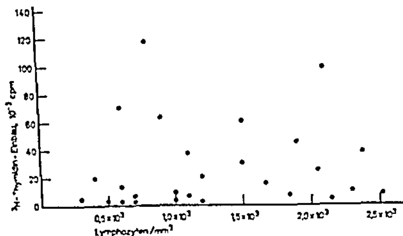


Abb 4 Abhängigkeit der Zinkstimulation *in vitro* von den absoluten Lymphozytenwerten des peripheren Blutes bei Patienten mit Lymphogranulomatose

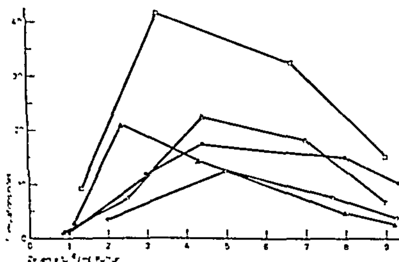


Abb 5 Zinkstimulation bei chronischer lymphatischer Leukämie in Abhängigkeit von der Zelldichte des Kulturansatzes. Dargestellt sind 5 Versuche mit 5 verschiedenen Patienten.

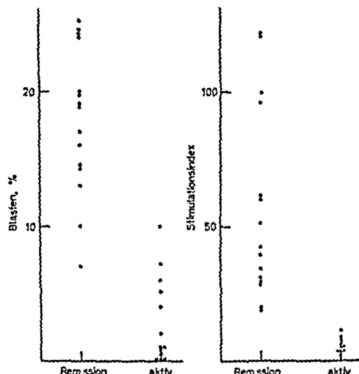


Abb 3 Zinkstimulation der peripheren Lymphozyten bei Patienten mit Lymphogranulomatose (^{3}H Thymidin Einbau und Prozentsatz Blasten) Gegenübergestellt sind Patienten in Remission und einem aktiven Erkrankungsstadium

Ergebnisse

Lymphogranulomatose Die Stimulation der Lymphozytenkulturen durch Zn^{2+} war bei Patienten mit Lymphogranulomatose gegenüber dem Normalkollektiv erheblich eingeschränkt Dies zeigte sich sowohl bei Messung des ^{3}H-Thymidin Einbaues (zwischen 1 343 und 94 124 cpm, Mittelwert 12 381 cpm) als auch bei der Bestimmung des Prozentsatzes blastenähnlicher Zellen (zwischen 0,0 und 24,3%) Kulturen von Normalpersonen wiesen dagegen zwischen 22 201 und 229 256 cpm (im Mittel 143 100 cpm) und 11,8–53,6% Blasten auf Diese Verhaltnisse sind in Abbildung 1 und 2 dargestellt.

Bei Patienten, die sich in Remission befanden, lag die Stimulation höher als bei Patienten in einem aktiven Stadium der Erkrankung (Abb 3), wenn auch noch erheblich unter der von Normalpersonen

Tabelle II Vergleich der Zinkstimulation bei Verwendung von autologem Plasma und homologem Plasma Gesunder. Der ^{3}H-Thymidineinbau wurde nach 144 h gemessen; angegeben sind mittlere cpm von Dreifachkulturen

Lymphogranulomatose		Chronische lymphatische Leukämie	
Eigenplasma	Normalplasma	Eigenplasma	Normalplasma
43 684	41 002	325	470
416	337	23 718	29 977
36 015	43 365	1 175	1 034
20 168	27 465	583	1 135
559	535	1 739	3 501

Einfluss von Plasmafaktoren. In einer Serie von Experimenten wurden Parallelkulturen von Patientenlymphozyten in Eigenplasma und in blutgruppengleichem Plasma gesunder Versuchspersonen kultiviert. Die dargestellten typischen Beispiele (Tab. II) belegen, dass die Stimulationsfähigkeit weder bei Lymphogranulomatosepatienten noch bei Patienten mit einer chronischen lymphatischen Leukämie gesteigert werden kann, wenn die Lymphozyten statt im autologen Plasma im Plasma gesunder Personen kultiviert werden.

Diskussion

Die Messung der Stimulation menschlicher peripherer Lymphozyten unter der Einwirkung mitogener Substanzen gilt heute allgemein als eine Methode, die immunologische Kompetenz dieser Zellen *in vitro* zu untersuchen. Die bei einigen lymphoproliferativen Erkrankungen mit Hilfe anderer Methoden festgestellten immunologischen Defekte finden ihre Erklärung in den Ergebnissen, die die stimulierte Lymphozytenkultur aufweist.

Die von uns im Modell der Zink-stimulierten Lymphozytenkultur gefundenen Ergebnisse bei Patienten mit Lymphogranulomatose und chronischer lymphatischer Leukämie stehen in Übereinklang mit den Befunden anderer Untersucher mit Hilfe anderer Stimulantien [3, 8, 11, 12, 15, 19, 24, 25]. Die herabgesetzte Stimulierbarkeit der Lymphozyten ist bei beiden Krankheitsbildern offensichtlich nicht durch Faktoren des Patientenplasmas bedingt, denn sie konnte bei Kultivierung der Zellen im Plasma gesunder Personen nicht verbessert werden. Dies entspricht

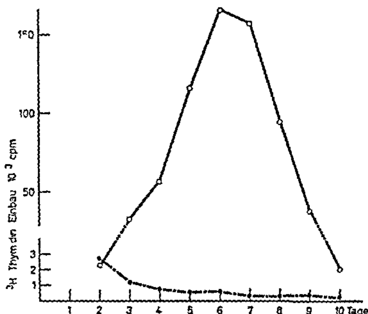


Abb 6 *In vitro* Stimulation der peripheren Lymphozyten durch Zn^{2+} bei einem Gesunden (O) und einem Patienten mit chronischer lymphatischer Leukamie (●) Der ³H Thymidin Einbau wurde in 24stundigen Abständen gemessen

Eine Abhangigkeit der Zinkstimulation von der bei vielen Patienten mit Lymphogranulomatose bestehenden Lymphopenie konnten wir bei den von uns untersuchten Personen nicht feststellen (Abb 4)

Chronische lymphatische Leukamie Die Unterschiede in der Transformationsfähigkeit der Lymphozyten im Vergleich zum Normalkollektiv waren bei dieser Patientengruppe noch grosser als bei den Lymphogranulomatosepatienten ³H-Thymidin-Einbau und Prozentsatz blastenahnlicher Zellen waren in Zinkkulturen kaum hoher als in unbehandelten Kontrollkulturen, wenn im Ansatz eine Zelldichte von $1{,}0 \times 10^6$ Leukozyten/ml bestand (Abb 1 und 2) Wurde allerdings die Zellzahl im Kulturansatz erhoht, so fand sich bei einer 5- bis 10fach hoheren Zelldichte ein deutlich grosserer Stimulationsindex (Abb 5), der jedoch noch weit unter dem bei Normalpersonen gefundenen lag Bei weiterer Erhohung der Zelldichte nahm der Stimulationsindex wieder ab Eine Steigerung der Stimulationsrate durch Verlangerung der Inkubation bis zu 240 h konnte nicht erreicht werden (Abb 6)

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ical state of the patient and to biochemical comparisons of normal and abnormal lymphocytes. Such studies are facilitated by freeze storage whereby limitations from ethical considerations, therapeutic measures and inconvenience to patients can be overcome. Lymphocytes may be stockpiled for future investigations. A set of conditions has been defined [THOMSON and O'CONNOR 1971] for freezing and thawing normal and CLL lymphocytes. The mortality rate, as assessed by cytological criteria, is negligible. In addition residual red cells contaminating the isolated lymphocytes are largely destroyed during freezing and thawing.

Lymphocyte ultrastructure before freezing and after thawing under these conditions has now been compared. Changes in ultrastructure and damage from ice crystal formation have been described in a number of previous reports on cryopreservation of cells and tissues [WEISS and ARMSTRONG 1960, TRUMP et al. 1965, STOWELL et al. 1965, FARRANT et al. 1967]. In the present study on lymphocytes, such changes were remarkably absent.

Material and Methods

The lymphocytes were separated from defibrinated blood from a healthy donor according to the method of THOMSON et al. [1970a] and WILSON and THOMSON [1968]. Lymphocytes were separated from blood from 3 CLL patients (one receiving chemotherapy, 2 untreated) by the same method but with omission of the passage through polystyrene beads designed to remove granulocytes. Granulocytes constituted less than 5% of the circulating white cells in these patients and abnormal lymphocytes, unlike the normal, may themselves adhere to polystyrene beads [THOMSON and ROBINSON 1967].

Preliminary studies in these cases showed that the proportion of abnormal (nucleolus containing) lymphocytes was 81 and 91% for the untreated patients and 95% for the treated.

The lymphocytes, suspended in medium TC 199 containing 8.3% (v/v) dimethyl sulphoxide (DMSO) and 15% serum, were cooled to -40 °C at a point freezing cooling rate of 1.5-3.4 °C/min and then immersed in liquid nitrogen (-196 °C). They were thawed at 37 °C at a rate of 30-40 °C/min. The full details of the method are given elsewhere [THOMSON and O'CONNOR 1971].

Frozen cells were stored at -196 °C for 14 weeks (normal lymphocytes) to 55 weeks (CLL). After thawing, cells were washed twice to remove DMSO, resuspended in TC 199 containing 5% ... serum and incubated at 37 °C for 1 to 2 h. The time elapsing between thawing and fixation varied from 3 to 5 h.

As a result of the ultrastructure procedures and for other reasons of technique, unfrozen lymphocytes were available for fixation within 5 h of blood collection. Their treatment during this period did not differ substantially from those prepared for freezing but they were not exposed to DMSO.

Acta haemat 46 338 348 (1971)

Preservation of Ultrastructure in Freeze-Stored Human Normal and Leukaemic Lymphocytes

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Abstract Normal human lymphocytes and lymphocytes circulating in 3 patients with chronic lymphocytic leukaemia (CLL-lymphocytes) were freshly isolated from defibrinated blood Suspensions were frozen stored at -196 C and subsequently thawed under specified conditions known to preserve well several aspects of behaviour *in vitro* Electron microscopy following short term culture showed no qualitative differences in ultrastructure between normal lymphocytes and abnormal CLL lymphocytes either before freezing or after this freezing and thawing procedure Microtubules were found in all types of cell

Key Words
Electron microscopy
Lymphatic leukaemia
Lymphocytes
Ultrastructure of lymphocytes

In health the small lymphocytes of peripheral blood (normal lymphocytes) are non dividing (diploid) cells which are heterogeneous in terms of origin life span and immune function The small lymphocytes circulating in increased numbers in chronic lymphocytic leukaemia (CLL) are also non dividing cells which for diagnostic purposes are indistinguishable morphologically from normal They are heterogeneous however, in terms of other criteria [Thomson *et al* 1966b] Certain lymphocytes circulating in CLL are ultra sensitive to the cytocidal action of colchicine *in vitro* and such cells have been designated abnormal lymphocytes [Thomson and Robinson 1967] The proportion of abnormal lymphocytes varies from case to case but in a series of 30 patients very high proportions of the increases in the number of lymphocytes are of this type [Thomson *et al* unpublished]

These findings have led to an examination of the relationship between the proportion of abnormal lymphocytes circulating and the clin

ical state of the patient and to biochemical comparisons of normal and 'abnormal' lymphocytes. Such studies are facilitated by freeze storage whereby limitations from ethical considerations, therapeutic measures and inconvenience to patients can be overcome. Lymphocytes may be stockpiled for future investigations. A set of conditions has been defined [THOMSON and O'CONNOR, 1971] for freezing and thawing normal and CLL-lymphocytes. The mortality rate, as assessed by cytological criteria, is negligible. In addition residual red cells contaminating the isolated lymphocytes are largely destroyed during freezing and thawing.

Lymphocyte ultrastructure before freezing and after thawing under these conditions has now been compared. Changes in ultrastructure and damage from ice crystal formation have been described in a number of previous reports on cryopreservation of cells and tissues [WEISS and ARMSTRONG, 1960, TRUMP *et al.*, 1965, STOWELL *et al.*, 1965, FARRANT *et al.*, 1967]. In the present study on lymphocytes, such changes were remarkably absent.

Material and Methods

The lymphocytes were separated from defibrinated blood from a healthy donor according to the method of THOMSON *et al.* [1966a] and WILSON and THOMSON [1968]. Lymphocytes were separated from blood from 3 CLL patients (one receiving chemotherapy, 2 untreated) by the same method but with omission of the passage through polystyrene beads designed to remove granulocytes. Granulocytes constituted less than 5% of the circulating white cells in these patients and 'abnormal' lymphocytes, unlike the normal, may themselves adhere to polystyrene beads [HUDSON and ROBINSON, 1967].

Preliminary studies in these cases showed that the proportion of 'abnormal' (i.e. colchicine ultrasensitive) isolated lymphocytes was 81 and 93% for the untreated patients and 95% for the treated.

The lymphocytes, suspended in medium TC 199 containing 8.3% (v/v) dimethylsulphoxide (DMSO) and 15% serum, were cooled to −49 °C at a post freezing cooling rate of 1.6–3.4 °C/min and then immersed in liquid nitrogen (−196 °C). They were thawed at 37 °C at a rate of 39–460 °C/min. The full details of the method are given elsewhere [THOMSON and O'CONNOR, 1971].

Frozen cells were stored at −196 °C for 34 weeks (normal lymphocytes) to 55 weeks (CLL). After thawing, cells were washed twice to remove DMSO, resuspended in TC 199 containing 5% v/v serum and incubated at 37 °C for 1 to 2 h. The time elapsing between thawing and fixation varied from 3 to 5 h.

As a result of the separation procedures and for other reasons of technique, unfrozen lymphocytes were available for fixation within 5 h of blood collection. Their treatment during this period did not differ substantially from those prepared for freezing but they were not exposed to DMSO.



Fig 1 Lymphocyte prepared after separation from healthy blood The micrograph shows a bilobed nucleus, infrequent mitochondria (M) and sparse endoplasmic reticulum Red blood cells (R) are often present in considerable numbers in preparations made without freezing and thawing

Cytological examination was carried out on smears fixed whilst wet (susa) and stained with Meyer's haemalum [TROWELL, 1955] prepared just after thawing or just after separation

For preparation for electron microscopy, all lymphocytes were suspended, at pH 7–7 5, in medium TC 199 with 30% v/v serum or in isosmolar buffered BSS with 10% v/v serum At least 15×10^6 cells, suspended in 0 5 ml medium were

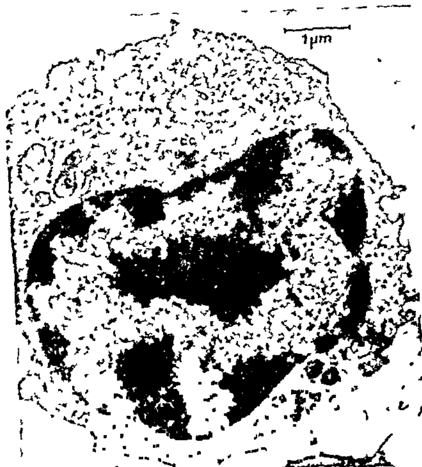


Fig. [illegible] Lymphocyte from chronic lymphocytic leukaemia prepared without freezing showing the cell centre (CC) situated in a concavity in the nuclear outline

mixed with 2.5 ml fixative solution containing 4% formaldehyde (freshly prepared) and 4% glutaraldehyde in 0.15 M cacodylate buffer (pH 7.4). The cells were fixed for 30 min at 37 °C. They were washed in 0.2 M cacodylate buffer at room temperature and postosmicated in 1% osmium tetroxide solution for 10 min. After washing in water the cells were resuspended in 3% gelatin solution at 37 °C and centrifuged. The gelatin was allowed to set at 4 °C and the pellet of embedded cells cut into small blocks. The blocks were dehydrated in methanol and embedded in Araldite. Thin sections were stained with lead citrate and uranyl acetate and examined with an AEI EM6 electron microscope.


1μm
G
Ce
3
M
G
M
4

Results

Light microscopy of smears of freeze-stored cells made just after thawing showed 4% dead cells consisting of intact or ruptured cells with pyknotic nuclei [THOMSON and O'CONNOR, 1971]. Some thawed lymphocytes classed as 'surviving' showed evidence of rupture or shedding of cytoplasm to a degree impossible to quantitate.

There were no consistent ultrastructural differences between the preparations from leukaemic and healthy blood whether unfrozen or freeze stored. Figures 1, 2 and 3 show cells prepared without freeze storage and figures 4, 5 and 6 show cells prepared after thawing. Ice crystal damage was not observed. There was no evidence of the cell rupture or loss of cytoplasm observed by light microscopy immediately after thawing. No change was observed in the nuclear membrane or endoplasmic reticulum, Golgi apparatus, lysosomes, ribosomes or microtubules.

Preliminary comparisons were made between lymphocytes of each type of preparation using quantitative methods to estimate the content of mitochondria and lysosome like bodies [LOUD, 1962; HALLY, 1964]. The results showed variations from cell to cell too great to permit analysis for differences between cells from different sources. All the lymphocytes are, therefore, described together.

The lymphocytes were roughly circular but the plasma membrane was very irregular in outline (fig. 1 and 2). There were numerous short processes and ruffles with some longer processes which in section sometimes appeared to be detached from the cell. In places long processes partially enclosed a droplet of the surrounding medium but there was no evidence that such droplets were engulfed by the cell. Small vesicles under the plasma membrane made it appear possible that micro-pinocytosis was occurring and that the plasma membrane was actively motile during life.

Although much was dependent on the plane of section in individual cells the proportion occupied by the nucleus did appear to vary. Many

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Fig. 1. Cell centre including centrioles (Ce) and Golgi apparatus (G) from a [illegible] CLL lymphocyte. The nucleus shows several profiles as the result of [illegible]

Fig. 2. Cytoplasm including mitochondria (M) and Golgi apparatus (G) from lymphocytes [illegible] blood thawed after [illegible] storage for 14 weeks.

1µm

G

Ce

3

1µm

M

G

M

4

cells possessed only a thin rim of cytoplasm. Others, possibly more frequently in leukaemic blood, possessed a higher proportion of cytoplasm associated with a kidney shaped or bilobed nucleus. In the majority of cells in all preparations, heterochromatin was well differentiated forming a peripheral condensation as well as a single mass centrally in the nucleus. Occasionally nuclear chromatin was much more homogeneous.

Where the nucleus was kidney-shaped, the cytoplasm at the concavity was occupied by the cell centre (fig. 3) consisting of the Golgi apparatus, centrioles and associated microtubules. The Golgi apparatus was predominantly vesicular in composition. In favourably orientated sections long microtubules could be seen radiating from the cell centre (fig. 6). There was no satisfactory way of comparing the numbers of microtubules in different cells but they were found in cells from leukaemic and normal, freeze stored and non frozen specimens.

Mitochondria occupied an average of 6% of the cytoplasm when measured by Hally's method for relative volume estimations. The figures obtained varied from 3–12%. The structure of the mitochondria is shown in figures 3 and 6.

Electron dense lysosome like bodies were observed at a frequency of approximately one for every three cells examined in each section. Once again no difference between populations was detected. The bodies were circular and about 1 μm in diameter, and some were highly electron dense. Smaller bodies and vesicles with electron dense contents as well as multivesicular bodies were found near the Golgi apparatus (fig. 3). Granular endoplasmic reticulum was present though only sparsely. There were numerous free ribosomes in the cytoplasm.

Discussion

Cryopreservation of lymphocytes under the present conditions [Thomson and O'Connor, 1971] induces a very low cell mortality. Cells not killed may display reduced initial capacity for respiration but

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Fig. 5. Part of a CLL lymphocyte thawed after freeze storage for [illegible] weeks. M = mitochondria.

Fig. 6. Freeze stored CLL lymphocyte with microtubules radiating from the [illegible]


1µm
5
0.5µm
6

Microtubules are a prominent feature of lymphocytes [ZUCKER-FRANKLIN, 1969]. They were found to be present in normal and leukaemic cells before and after freeze storage but have not been studied quantitatively. It seems likely that microtubules would be damaged by freezing and thawing but changes in them are often reversible [BRINKLEY *et al.*, 1967]. Two hours in culture might provide time for their recovery. It is important to establish the presence of microtubules because of the difference in colchicine sensitivity which persists in freeze stored lymphocytes [THOMSON and O'CONNOR, 1971].

Colchicine is known to affect microtubules [MALAWISTA and BENSCH, 1967; MALAWISTA, 1968]. The degree to which tissues bind colchicine can be correlated approximately with their microtubule content [BORISY and TAYLOR, 1967]. It is of interest to note, therefore, that normal and abnormal lymphocytes exhibit a grossly different sensitivity to the cytocidal action of colchicine at a concentration of 10^{-7} M, but their capacity to bind colchicine at concentrations of this order is very similar [THOMSON *et al.*, 1971]. In respect of microtubules, on a qualitative level there appeared to be no difference between leukaemic and normal lymphocytes before or after freeze storage.

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Acta haemat. 46: 349-361 (1971)

Cytokinetics of Bone Marrow Stroma Cells after Stimulation by Partial Depletion of the Medullary Cavity[1]

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Key Words
Autoradiography
Bone marrow regeneration
Bone marrow stroma
Rat bone marrow

Abstract. The continuous administration of tritiated thymidine (^3H-TdR) into pregnant rats resulted in a complete labelling of cell nuclei in the offspring. Further injections with ^3H-TdR for 6 weeks maintained the 100 percent labelling. Six weeks after the last ^3H-TdR application, only cytokinetically resting cells were selectively labelled in the bone marrow of these animals. These comprised reticular cells, endothelial cells and 5% of the bone marrow lymphocyte population.

In these animals a standardized amount of the marrow of both the femora was removed by mechanical means. In the undepleted part of the femur a stimulation of formerly cytokinetically resting cells could be observed. There was no indication of stimulation in distant bone marrow parts.

In an additional experiment in which a single injection of ^3H-TdR was given at various intervals after depletion, the above findings were supported by a high uptake of ^3H-TdR in the stroma cells of the bone marrow. The findings indicate that repopulation of bone marrow stroma cells is a necessary process for a restoration of haemopoiesis.

In previous work of our group [8-10] evidence has been provided that certain cell types of the rat bone marrow retain tritiated thymidine (^3H-TdR) for weeks and months after this substance has been administered in utero and during neonatal life. These cytokinetically resting cells can be morphologically identified as reticular cells of various kinds, endothelial cells, and a small fraction of bone marrow lymphocytes. The

[1] Research supported by the European Atomic Energy Community (Euratom Contract No. [illegible] BIAC) and the Bundesministerium für wissenschaftliche Forschung.

term 'reticular cells' is used for cells forming the stroma network of the bone marrow Some of these cells apparently have phagocytic activity The term endothelial cells is meant for both the sinusoidal lining cells and the true vascular endothelial cells (for detailed description see elsewhere [9, 11]) It was shown that after administration of X-irradiation or cytotoxic agents [1, 10, 13] during the regeneratory process the fraction of small mononuclear cells called 'resting bone marrow lymphocytes' could be stimulated to proliferate However, there was no evidence for a stimulation of reticular or endothelial cells demonstrable in these investigations It is the purpose of this study to extend our previous observa tions of the cytokinetic properties of slowly turning over bone marrow cells by the application of an adequate stimulus for proliferation of reticular and endothelial cells The model of MALONEY and PATT [17–19] provided a convenient technique to study regeneration of bone marrow after mechanical eradication However, these authors were mainly interested in the cells which are able to regenerate the bone marrow parenchyma since in this model the bone marrow cavity was completely evacuated In the present study the aim was to remove only a standardized part of the femoral bone marrow in order to evaluate (a) a possible stimulation of stroma cells in the undamaged part of femur bone marrow and (b) a possible stimulation of such cells in distant bone marrow parts under these experimental conditions

Material and Methods

Animals The experiments were carried out with 12 week old rats of the inbred strain LEW F 134+4 (Wistar Institute) Two groups of animals were injected with ^{3}H TdR [2] specific activity 2 0 Ci/mM as follows

Group I (single injection series) Rats of this group received 1 μCi ^{3}H TdR/g body weight intraperitoneally 1 h before being sacrificed The group included 16 animals weighing 140–160 g Two animals at each point were sacrificed 6 h to 30 days after depletion of the bone marrow Two controls were used in which the bone marrow was not depleted This series permitted evaluation of the rate of proliferation of bone marrow stroma cells at various time intervals after marrow depletion by measuring the labelling index of these cells [6]

Group II (continuous infusion series) Rats of this groups were the offspring of Wistar rats which had been continuously infused with ^{3}H TdR from day 9 of pregnancy until delivery [11] The approach used for continuous labelling of rats was a modification of that described by LITTLE *et al* [16] The amount of

[2] ^{3}H TdR was obtained from the Radiochemical Centre Amersham U K

^{3}H-TdR administered per day was 1.6 μCi/g body weight. 100% labelled newborn rats were subsequently injected every 12 h s.c. with ^{3}H-TdR at a dose level of 0.8 μCi/g body weight/day for 6 weeks after birth. Six weeks after the last ^{3}H-TdR injection (i.e. 12 weeks after birth) only cytokinetically resting bone marrow cells were still ^{3}H-labelled. The group included 21 animals weighing 150–160 g each. Two animals at each point were sacrificed 6 h to 30 days after depletion of the bone marrow. The marrow of 3 controls was not depleted. This series provided information on the proliferative activity of bone marrow stroma cells by evaluation of the decrease in labelling index and labelling intensity of these cells.

Removal of marrow. In both groups a standard sized amount of marrow of both the femora was removed by the following method. Rats were anesthetized with ether and an incision was made at the knee joint. The joint was opened. With a dental drill a small hole was made through the epiphysis of the femur in order to expose the bone marrow cavity. Then a Klima-Rosegger bone marrow aspiration needle was used to remove a 1.5-cm long bone marrow cylinder with a diameter of 1.3 mm.

Histological and cytological preparations. Cytological smears of bone marrow from the right femur and tibia were prepared. The left femur and tibia were observed for histological sections. Autoradiographs of the smears were made using Kodak AR 10 stripping film and were exposed for 45 days. Autoradiographs of histological sections were prepared by the dipping technique using Ilford L4 liquid emulsion. The exposure time was 60 days. After photographic processing the smears were stained with Giemsa at pH 5.75 and histological sections with haematoxylin-eosin. All autoradiographs, including controls, were prepared at the same time.

Evaluation. In the autoradiographs of smears at least 700 stroma cells per animal were evaluated as labelled or unlabelled (background 3 grains/cell) to determine the labelling index. For small lymphocytes, the labelling index was determined from at least 300 cells. The stroma cells were differentiated in reticular and endothelial cells. In group II grain counts were made over at least 50 labelled cells of reticular cells A, reticular cells B, endothelial cells and small lymphocytes to determine the mean grain count.

In the histological sections of group II the number of nucleated cells and the number of labelled cells per mm² bone marrow were determined according to the method of Sasaki [?] and Cr[?]ns [11].

Results

Bone marrow of the control (group III). The evaluation of bone marrow cells at the time of the beginning of the depletion experiment showed that the ^{3}H-labelling procedure had resulted in a selective labelling of cytokinetically resting bone marrow cells as found in previous experiments. The cells which at that time still appeared to be labelled were endothelial cells, reticular cells type A and B and a small fraction



of bone marrow lymphocytes (morphological classification see elsewhere [9, 11]). When in histological sections 7,000 nucleated cells/mm² were counted, 270 labelled cells/mm² were found, i.e. a labelling index of 3.9%. In bone marrow smears of the same group 4.3% of labelled cells were found. Therefore, a differentiation of all labelled cells from the smears into the described morphological classes seems justified. This amounted to 26% reticular cells type A, 30% reticular cells type B, 19% endothelial cells and 25% labelled lymphocytes. The mean grain counts after an exposure time of 45 days were 27 grains/cell for reticular cells type A, 32 grains/cell for reticular cells type B, 38 grains/cell for endothelial cells and 19 grains/cell for lymphocytes.

General renewal of the cellular stroma and the bone marrow after depletion. The reconstitution of the depleted bone marrow was essentially similar to the findings extensively described by STEINBERG and RÖHLICH [20, 22]. In the depleted bone marrow part during the first 2 days after removal of the tissue cylinder a blood coagulum was found in which mature granulocytes and lymphocytes were present. The undisturbed bone marrow amounted to ¹/₄ of the former marrow and a sharp border to the blood coagulum could be seen in all preparations during that time. At the endosteal layer in the region of the depleted part, mainly mesenchymal cells could be detected, some of which showed the characteristics of osteoblasts (fig. 1). During the first week primitive mesenchymal cells from the endosteum and from the remaining bone marrow in the distal part of the femur repopulated the depleted cavity (fig. 2 and 3). In some parts a network of fibrocytic cells could be seen. Bone trabeculae and capillaries were present again 7–9 days after depletion. It was of interest to note that also many lymphocyte-like cells were observed in the primitive marrow.

The entire regenerative process was very similar to the pattern seen in a developing bone marrow of the rat [5]. Haemopoietic cell clusters were first seen in the depleted region 5 days after the beginning of the experiment. The histological picture observed 30 days after depletion showed bone marrow formation in which an increased number of fat spaces was seen, as illustrated in figure 4.

Primitive mesenchymal cells repopulate the depleted cavity. Small capillaries are present with mature leukocytes. Lymphocyte-like cells can be seen scattered through the mesenchymal cells. [illegible] Haematoxylin-eosin [illegible]

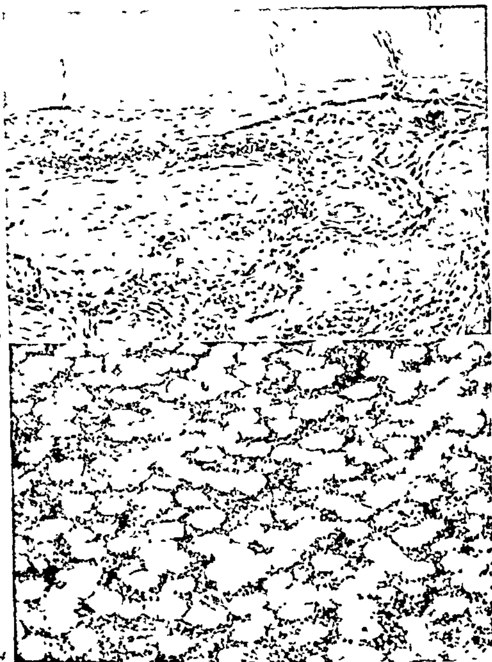


Fig. 3 Histological section of femoral bone marrow 9 days after partial depletion. Primitive mesenchymal cells arising from the endosteum of the corticalis and the bone trabeculae extend into the marrow cavity. Haematoxylin eosin, × 70

Fig. 4 Histological section of femoral bone marrow. Regeneration 30 days after partial depletion. The formerly depleted part is almost completely filled but an increased number of fat spaces as compared to normal rat bone marrow can be noted. Haematoxylin eosin, × 70

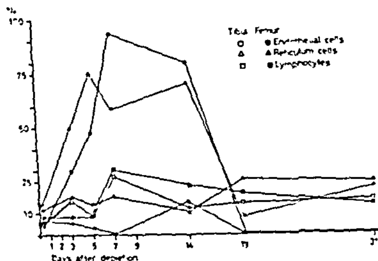


Fig. 5. Changes in labelling index as a function of time over endothelial cells, reticular cells and lymphocytes after partial depletion of femoral bone marrow. Flash labelling experiment group I

Proliferative behaviour of stroma cells in group I (flash labelling). In the undepleted region of femoral bone marrow, i.e. the proximal part of the femur the labelling index of stroma elements of the bone marrow was markedly changed as shown in figure 5. As can be seen the percentage of labelled cells increased to maximal levels between 5–14 days after depletion, where over 70% of all reticular and endothelial cells were labelled in the smears. After the 14th day a decrease of labelling index was observed so that by day 19 the values were nearing those of the controls, where about 10% of these cells were found labelled. Lymphocytes showed also an increase in the labelling index with a peak at day 7, at which time about 30% were labelled as compared with about 8% in the controls. Figure 5 also indicates the labelling index of the stroma cells in the tibiae of the same animals. Here it was found that despite some variations in the labelling index of reticular and endothelial cells, no definite increase or decrease could be demonstrated. However, the small lymphocytes in the tibiae of these animals showed a very similar labelling pattern to that found in the femora, i.e. an increase of labelling index at day 7.

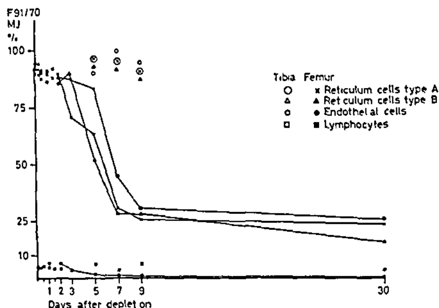


Fig 6 Changes in labelling index as a function of time over endothelial cells reticular cells and lymphocytes after partial depletion of femoral bone marrow Continuous labelling experiment group II

Proliferative behaviour of stroma cells in group II (100% labelling) The change in labelling index at different times after depletion of rats which received ^{3}H-TdR continuously until 6 weeks of age is shown in figure 6 In the smears, about 90% of reticular cells and endothelial cells were labelled at the beginning of the experiment, when the animals were 12 weeks old 5% of the entire lymphocyte population were also still labelled Following depletion of the marrow, a remarkable decrease of labelling index could be seen in the proximal undepleted part of the femur starting at day 3, so that by 7 days more than 60% of the stroma cells had lost their labelling, indicating a high proliferative activity As also indicated no such changes in the labelling index could be noted in the respective cells of the tibiae

The examination of histological sections by means of the Sandkühler method also demonstrated a reduction in the number of labelled cells Three days after the start of the experiment 93% of the original value of labelled nucleated cells/mm^2 bone marrow was found, at 5 days this was reduced to 51% and at 7 days only 41% remained la-

could be excluded [7, 8, 12] the proliferative activity of the labelled cells can be estimated by evaluation of the decrease in labelling index or and labelling intensity

The results of experiments of group II could be interpreted as meaning that all cells physiologically at rest were stimulated since a decrease of labelling intensity beyond day 7 was found over all cells. Additional support for this interpretation was also obtained by the pulse labelling experiment (group I) since over 70% of the entire population was found to be labelled between day 5 and day 14 after depletion. It is of interest to note that in the proximal femoral marrow no topographical difference in the change of labelling pattern could be detected. On the other hand in agreement with the findings of MALONEY and PATT [17–19] there was no indication for a stimulation of stroma cells in abscopal bone marrow as measured by the labelling pattern of these cells in the tibia.

As far as the labelled fraction of small bone marrow lymphocytes (in group II) is concerned the findings indicate a stimulation to proliferate. There was also some indication that in the unaffected tibia of the animals these cells were also stimulated. These results are comparable with our earlier investigations of the labelling pattern of this cell class during recovery of bone marrow cellularity after X-irradiation or cytotoxic agents. A possible explanation for the cytokinetic behaviour of these cells preceding the final haemopoietic regeneration was that these cells play an important role in the recovery of haemopoiesis as has been studied extensively previously [1, 13].

Considering all the data it can be speculated that stroma cells of the bone marrow are able to initiate restoration of a depleted bone marrow matrix by proliferative activity thereby providing the necessary prerequisite for seeding of haemopoietic cells. The origin of the latter cells cannot be answered by the present data.

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... experiment, and 6 days later very low levels

Discussion

...arrow cellularity has been investigated by sev... ...ve studies [2, 3, 17, 20, 22]. The mechanisms ... neonatal development of bone marrow where ... involved. One aspect is the repopulation of ... s found by the experiments of KNOSPE *et al* ... for repopulation of haemopoiesis. This process ... m... ocirculation and the existence of a reticular ... sary for seeding of transplanted bone marrow ... ...t is the origin of the haemopoietic cells which can ... week after depletion to be again present in the ... our exp... riments the morphological findings showed ... ...storation of a primitive bone marrow took place before haemopoietic cell clusters could be detected. It could be shown that primitive mesenchymal like cells repopulated the depleted area arising apparently both from the undamaged part of the femoral bone marrow and the endosteal layer. Whether mesenchymal cell elements in the adjacent bone contribute to the restoration of bone marrow stroma as suggested by MA... and HART [18] is hard to say from our experiments. However, there is suggestive evidence of a proliferation and a possible immigration of cells from Haversian canals during the initial phase of restoration (fig. 3).

It was expected that by the method used new information could be obtained about an adequate stimulus for proliferation of stroma cells which under normal steady state conditions are cytokinetically at rest [4, 11, 14]. As already mentioned, 2 particular questions were of interest in our investigations: (1) Is it possible to trigger resting stroma cells of the bone marrow to proliferate by partial depletion? (2) Is any stimulating influence measurable in abscopal bone marrow areas after femoral bone marrow depletion? For these specific questions the method of complete ^{3}H TdR labelling of bone marrow seemed a convenient model. This method results after a suitable time period between observation and the last ^{3}H TdR injection in a selective ^{3}H labelling of stromal elements of the bone marrow. Since radiotoxic effects from the dose administered

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J H THOMAS D E B POWELL **Blood Disorders in the Elderly**

Wie aus dem Vorwort zum vorliegenden Buch hervorgeht gaben praktische Gesichtspunkte den Anstoss zu seiner Abfassung Zwar gibt es sowenig Alterskrankheiten des Blutes wie es sonst spezifische Alterskrankheiten gibt Geriatrie ist aber in den letzten Jahren aus vorwiegend äusseren Gründen eine Spezialität geworden welche die Verbindung zu anderen Zweigen der Medizin zu verlieren droht Das Buch will eine Brücke zwischen Geriatrie und Hamatologie schlagen Es richtet sich weniger an den Hamatologen als an den geriatrisch interessierten Allgemeinmediziner Allerdings wird auch der Hamatologe eine Fulle von wichtigen und nutzlichen Daten und Feststellungen finden Ihm sei besonders die Lektüre des Einführungskapitels empfohlen in welchem gezeigt wird dass hohes Alter an sich mit keinen Veranderungen des Blutes einherzugehen braucht Wie ein roter Faden zieht sich sodann der Hinweis auf das haufige gleichzeitige Vorkommen mehrerer Krankheiten im Alter durch das Buch Mit Recht werden die in Hamatologielehrbuchern oft nur am Rande berücksichtigten sekundaren Blutsveränderungen bei Allgemeinkrankheiten (Infektionen Neoplasien usw) an den Anfang gestellt Besonders eingehend wird sodann der Eisenmangel besprochen stellt er doch nicht nur ein wichtiges medizinisches sondern auch ein soziales Problem im hohen Alter dar Weitere Kapitel sind den megaloblastaren Anamien den Tumoren des lymphatischen Systems den Leukamien den Dys und Paraproteinämien den myeloproliferativen Storungen den hamorrhagischen Diathesen den hamolytischen Anamien und der Knochenmarksinsuffizienz gewidmet Stets wird der klinischen Diagnose aber auch der Therapie ein breites Feld eingeraumt Besondere Beachtung verdient das Kapitel über die Vorbeugung von (Mangel) Anämien Das Buch ist in einem flussigen nicht immer sehr konzisen Stil geschrieben Kurze typische Krankengeschichten dienen zur Illustration und Auflokkerung des Textes Als kleinen Schonheitsfehler empfand der Referent dass die Hamoglobinwerte bald in g/100 ml bald in % angegeben sind wobei es dem kontinentalen Leser überlassen bleibt zu merken dass in England 100% Hamoglobin einer Konzentration von 14 6 g/100 ml entspricht U BUCHER *Bern*

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